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PHYSIOLOGICAL ZOÖLOGY

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PHYSIOLOGY OF DEVELOPMENT OF THE FEATHER. VI. THE PRODUCTION AND ANALYSIS OF FEATHER-CHIMAERAE IN FOWL[1]

(Four text figures; five plates)

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I. INTRODUCTION

THE present study takes its departure from the preceding paper of this series (Lillie and Wang, 1941), in which the mechanism of development of the feather papilla into the definitive feather was analyzed experimentally. In that paper it was shown, among other things, that feather-chimaerae could be produced by combining contralateral halves of a breast papilla and a saddle papilla within the follicle of one of them (L/R chimaerae). The feather so produced was intermediate in length and form between typical breast and saddle feathers, but each half retained the proportional lengths of fluff and vane, distribution of barbules in the vane, and the type of pigmentation char-

[1] This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

acteristic of its own tract (Pl. I, Fig. 7). The analysis was not carried further.

It was obvious, however, that the subject presented unrealized possibilities for further analysis of the mechanism of development of the feather. Accordingly, additional experiments were made in which dorsal and ventral halves of breast and saddle papillae were recombined (D/V chimaerae). The results furnish a critical test of our theories of development and present some new problems.

In the preceding study it had been shown that, whereas isolated lateral or dorsal halves of papillae could produce complete feathers, the characteristic product of a ventral half lacked a rhachis and the barbs that developed formed a radially arranged tuft with a common base. Would the combination of dorsal and ventral halves, dissimilar with respect to not only origin but also potency, form a single feather? And, if so, what would be its characters? In the bilateral combinations (L/R) each component is totipotent. In the dorsoventral combinations only the dorsal component is totipotent. In the L/R experiments only one type of combination is possible; in the D/V combinations there are two types: breast dorsal and saddle ventral (B/S), and vice versa (S/B).

From the new experiments chimaerae of four main types were derived: (1) longitudinal,[2] (2) transverse, (3) pseudobilateral, and (4) twins.

An example of the marginal type is shown in Plate II, Figure 8. The apex is breast-type, but the margins of the feather on both sides below the apex are saddle-type, and the center is breast-type. The fluff is of approximately equal length on the two sides. Such feathers are derived only from B/S combinations.

Examples of the transverse type are shown in Plate III, Figures 9 and 10. The apex is saddle-type and the base breast-type, though the example shown in Figure 10 is very asymmetrical. The fluff is slightly unequal on the two sides in asymmetrical examples.

Examples of the pseudobilateral type are shown in Plate IV, Figure 11 (S/B derivation) and Figure 12 (B/S derivation). In both cases one side of the feather is predominantly breast with longer fluff and the other predominantly saddle. As will be shown later, the pseudobilaterals of B/S and of S/B derivation differ from one another and also from the true bilaterals of L/R derivation in important respects.

An example of twin chimaerae is shown in Plate V (Fig. 13). They are conjoined at the base, and each partner is itself a chimaera.

The experiments were all performed on Brown Leghorn capons. Examples of the normal breast and saddle feathers such as were used in the experiments are shown in Plate I, together with a bilateral chimaera produced by an L/R combination in previous experiments (cf. color plate, Lillie and Wang, 1941).

The significant characters used in the present account are as follows:

1. The feather as a whole (cf. Pl. I, Figs. 5 and 6).
   a) Total length: the typical breast feather is about 100 mm. and the typical saddle feather about 150 mm. long in the parts of the tracts used.
   b) Fluff ratio: this is the product of total length divided by length of fluff. The fluff ratio in the breast is about 2 and in the saddle about 3; the absolute length of fluff is about the same in the two tracts.
   c) Form of the feathers: breast, relatively

[2] An alternate designation is "marginal," indicating that the right and left margins of the vane represent one component and the central part and rhachis the other component.

broad with rounded apex; saddle, relatively narrow with more acuminate apex.

d) Growth rates: faster in the breast than in the saddle.

2. Parts of the feather.

a) The vane.

i) Pigmentation: black in the breast with iridescence; golden brown in the lacy margins, brown in the central web of saddle feathers. Colors are due to melanophores.

ii) The barbs of breast feathers carry barbules along their entire length, which interlock to form a coherent web. In saddle feathers the barbs carry barbules and form a web only in their basal portions; the margins of the vane are "lacy"; the web broadens from the apex toward the base of the feather (Pl. I, Fig. 6).

b) The fluff. Apart from greater length of barbs in the breast, the characters of the fluff are very similar in both tracts.

These distinguishing characters constitute the criteria by which the B and S components of chimaera feathers are diagnosed and their reciprocal effects determined.

The characters of the chimaerae as a whole furnish valuable criteria of the quantitative amounts and bilateral distribution of B and S components: the length is always intermediate, and the axis always straight, no matter how unequal the distribution of B and S on the two sides. Growth rates are always the same on the two sides. Fluff ratio, however, is determined separately on the two sides by the proportionate amounts of the components.

Pigmentation and the distribution of barbules on the barbs are indispensable characters in determining the quantity and distribution of B and S components. In general we regard the components as self-differentiating; if this were strictly so, there would be no difficulty in determining the exact distribution of components in chimaerae; but discrimination is occasionally difficult, owing especially to substitution of brown for black pigment (p. 18).

II. ANALYSIS OF DEVELOPMENT OF THE CHIMAERAE

1. TECHNIQUE OF EXPERIMENTS[3]

The general method of operation used in recombining the dorsal and ventral half-papillae from two tracts is the same as the recombinations of sagittal half-papillae which yielded the bilateral chimaerae (see Lillie and Wang, 1941, p. 118). In the present experiments three old capons of over two years of age and four young capons six months old, which had been castrated at the age of six weeks, were used.

The left breast and the extreme posterior saddle tract on the same side were plucked about twenty days before an experiment, in order to have regenerating feathers with active papillae available. The bird was then fastened on the operating board on its right side and thoroughly anesthetized by intraperitoneal injection of Nembutal (pentobarbital sodium, Abbott), about 0.5 cc. for each kilogram body weight. In each experiment 10 combinations were made, 5 B/S and 5 S/B. All combinations were autoplastic. Seven experiments were performed altogether: in 5 of them the breast was chosen as the site of combination, and in 2 the saddle (Table 1).

The usual aseptic precautions were taken, and the operations were made under the binocular microscope. If the breast were to be the site of combination, 5 of the regenerating feathers in the saddle were plucked in order to use their papillae for transplantation. The follicles were then slit open by dorsal incisions to

[3] For a general orientation in the technique of operating on papillae consult Lillie and Wang, 1941, p. 109.

the base, with care to avoid touching the papilla. This was then brought into focus under the microscope by fine pointed forceps without touching the papilla itself; with iridectomy scissors the papilla was divided into dorsal and ventral halves by a single cut. The halves were then detached, leaving sufficient wall of the follicle and subjacent derma for purposes of handling, and placed in separate dishes of sterile Ringer's solution. This was repeated five times, until one dish contained 5 dorsal halves of saddle papillae and the other 5 ventral halves. All faulty cases were discarded.

The breast follicles were then prepared to receive saddle transplants with precautions similar to those used for the donor papillae. Ten of the regenerating feathers of the breast tract situated near its middle were plucked from adjacent follicles in two horizontal rows, 5 in one row and 5 in the next row ventral. The dorsal row was intended to receive the dorsal halves of saddle papillae (S/B, numbered 1–5) and the ventral row the ventral halves (B/S, numbered 6–10); the dorsal halves of the papillae of the dorsal row were removed, leaving the ventral halves in place, and the reverse was done in the ventral row; the combinations S/B and B/S were then made. After each combination is made, the flaps of the open follicle are brought together; healing is rapid.

When the saddle was used as host tract the above procedures were reversed so that the dorsal row was still S/B and the ventral B/S.

In making the combinations each host follicle was treated separately from plucking to completion, one after the other. When the host half is ventral, the placing of the dorsal donor half upon it is relatively simple, as accurate fitting is made in full view; but, when the dorsal half of the host papilla is preserved, it conceals the donor ventral half from view during placing, and the fitting is thus liable to be less accurate. This is reflected to some extent in the results (see Tables 1 and 2). Fusion of the components was facilitated by keeping the site as dry as possible, free from blood or Ringer's solution, for a considerable time. For this purpose the bird was kept motionless upon the operating board for several hours, or even overnight if the operation was performed late in the day. The birds were then kept separately in isolation cages for at least a week, and in the case of the older birds, permanently. The host follicles were marked for identification by clipped feathers or tattooing.

The papillae consist in all cases of a half which retains all its original connections (*host half*) and a half which has been isolated and transplanted (*donor half*). The latter is presumably in a less favorable condition for development, owing to the necessity of acquiring vascularization from the host.

2. TABULATION OF RESULTS

Table 1 contains a classified list of the chimaerae produced. Experiments 89, 90, and 91 were performed in February, 1941; after results had begun to appear, experiments 301, 302, 303, and 304 were performed in May, 1941. Altogether, 70 combinations were made (10 in each experiment), from which 29 chimaerae were produced. Each number entered under "Chimaerae Produced" in the table stands for an individual feather.

It is to be noted that marginal chimaerae occur only in B/S combinations and transverse chimaerae only in S/B combinations—a result discussed in the next section. S/B combinations succeeded only on the breast tract; in B/S combinations 7 out of 10 produced marginal chi-

maerae on the saddle tract and only 5 out of 25 on the breast tract. This is to be regarded as a result of the greater ease in making S/B combinations on the breast and B/S combinations on the saddle.

Table 2 accounts for the operations that failed to produce chimaerae. In 12 cases no feathers resulted. In other operations that failed to produce chimaerae the feathers produced were either breast- or saddle-types due to failure of one component to develop; this was always the donor component, so that breast feathers occurred only in the breast tract and saddle feathers only in the saddle tract. The donor component, which is a graft, simply failed to "take" in these cases or degenerated. The host component may be either a dorsal half or a ventral half, so that the results are similar to defect experiments of our previous paper (1941) and are so classified. The percentage column exhibits very clearly the greater difficulty of operations in the saddle tract.

TABLE 1*

DERIVATION AND CLASSIFICATION OF B/S AND S/B CHIMAERAE

Classification of all feather-chimaerae from D/V combinations with reference to type of experiment, site of host tract, and percentage of successful operations. The number in the first column designates the experiment; the numbers under "Chimaerae Produced" identify 1 each of the 10 combinations made in each experiment.

Experiments				Chimaerae Produced			
Number	Type	Site	No. of Combinations	Longitudinal (Marginal)	Transverse	Pseudo-bilateral	Twins
89	S/B	Breast	5	.....	No. 1	.....	No. 3
90	S/B	Breast	5	.....	.....	Nos. 1, 2, 3	No. 4
91	S/B	Breast	5	.....	.....	No. 4	.....
301	S/B	Breast	5	.....	Nos. 2, 3	.....	No. 5
302	S/B	Saddle	5	.....	.....	.....	.....
303	S/B	Saddle	5	.....	.....	.....	.....
304	S/B	Breast	5	.....	Nos. 1, 5	.....	.....
89	B/S	Breast	5	Nos. 8, 9	.....	.....	No. 7
90	B/S	Breast	5	.....	.....	No. 9	.....
91	B/S	Breast	5	No. 7	.....	.....	.....
301	B/S	Breast	5	Nos. 8, 9†	.....	.....	.....
302	B/S	Saddle	5	Nos. 10, 6†, 7†, 8†	.....	.....	.....
303	B/S	Saddle	5	Nos. 8, 9, 10	.....	.....	.....
304	B/S	Breast	5	.....	.....	Nos. 7, 8, 9	.....
Totals	.....	.....	70	12	5	8	4

* Successful incorporation = 41 per cent (29 out of 70 cases).

† These feathers are atypical, predominantly breast-type, but with a narrow marginal saddle stripe on one side or on both (for discussion see p. 8). They are not included in Table 3.

3. THEORETICAL CONSIDERATIONS

In both B/S and S/B experiments the expectation would be the production of symmetrical chimaerae if the respective components were strictly dorsal and ventral halves harmoniously combined. But both kinds of combinations yield a large proportion of asymmetric feathers. The reasons for this appear to be (1) deviations from the strictly frontal plane in

dividing the papillae, (2) the difficulty of exact adjustment of components in the recombination, and (3) slipping of components after recombination. But the fact that a single feather usually results shows considerable capacity for regulation of faulty unions. We have not been able to observe directly the healing-together of components.

In each combination both a dermal and an epidermal constituent is involved. The former determines by induction the axis and dorsal surface of the feather (cf. Lillie and Wang, 1941, p. 130)—a conclusion subsequently confirmed by Wang's experiments on transplantation of papillae denuded of ectoderm (Wang, 1943). The feather itself is entirely epidermal in origin. Breast ectoderm determines only breast characters and saddle ectoderm only saddle characters, irrespective of the source of the dermal part of the papilla. The latter point has been demonstrated conclusively by Wang's experiments on transplantation of papillae denuded of ectoderm between breast and saddle tract (Wang, 1943): whereas *intact* papillae produce feathers with all the characters of the donor tract when transplanted to the other tract (i.e., saddle feathers in the breast and breast feathers in the saddle), *denuded* papillae acquire ectoderm from the host follicle and produce feathers of the type of the host tract. The dermal part of the papilla is entirely devoid of tract specificity.

TABLE 2

RESULTS OF OPERATIONS THAT FAILED TO PRODUCE CHIMAERAE

Classification of all combinations in which feather-chimaerae were not produced.

	On Breast: Breast Feathers			On Saddle: Saddle Feathers			Percentage*	
	S/B	B/S	Total	S/B	B/S	Total	On B	On S
Failed	2	1	3	7	2	9	6	45
Normal	0	5	5	0	0	0	10	0
Slightly defective	7	2	9	1	0	1	18	5
Half-vane	2	2	4	0	0	0	8	0
Tuft	3	1	4	2	1	3	8	15
Dwarf	0	2	2	0	0	0	4	0
Anomalous	1	0	1	0	0	0	2	0
Total	15	13	28	10	3	13	.....	.....

* The percentage is calculated in each case on the basis of the total number of combinations made on breast (50) and on saddle (20).

A. THE DERMAL COMPONENTS

The fundamental fact with reference to the production of chimaerae, excepting the twins, is that the two united components act as a unit in the induction of a single dorsal field in the ectoderm. The clue to this condition is found in our previous determination (Lillie and Wang, 1941) that the ventral half of the papilla produces tuft feathers consisting of a radially arranged series of barbs without a rhachis; it contains no part of the dorsal inductor. The dorsal half, on the other hand, produces a complete and typical feather; it contains all of the dorsal inductor. The induction field of the papilla was regarded as spread over the entire dorsal surface, not confined to a narrow strip corresponding in extent to the rhachis; the reason for the latter conclusion was that "ventral" halves frequently produced half-vane feathers, presumably because a strip of the dorsal inductor had been included in it.

As shown by the normal development, the dorsal half of the papilla produces, first, a series of parallel barbs, and the rhachis arises later beneath the bases of these barbs in the center; tangential movements of barbs then begin, and the barbs unite seriatim with the outgrowing rhachis (Lillie and Wang, 1941, p. 106). The location of the rhachis is thus determined secondarily within the dorsal field, which produces the apex of the feather.

It would accordingly follow in the production of the chimaerae that, if both

components were accurately cut and fitted, the dorsal field would form in the center of the dorsal component, whether B or S, and that the ventral component would conform to this arrangement and symmetrical marginal and transverse chimaerae would be derived. However, if the dorsal component were not a true dorsal half, the dorsal field would be induced excentrically; from various degrees of such excentricity all grades of asymmetrical chimaerae up to pseudobilateral would be derived. Inexact adjustment of components or secondary slipping after adjustment might contribute to other abnormalities often to be noticed in the chimaerae.

Turning now to the ventral component: if it were not cut accurately and contained a sufficient part of the dorsal field, it would form an independent rhachis excentrically placed, which, together with the rhachis formed in the dorsal component, would result in the formation of conjoined twins (Pl. V, Fig. 13). Only 4 cases in all were produced (Table 1). Adding the independent variabilities of dorsal and ventral components, the twins should be still more variable in composition than the single chimaera, but each partner should show chimaera characters, which is, indeed, the case in our 4 examples.

B. THE EPIDERMAL COMPONENTS

The previous studies of feather development have shown that all loci of a feather may be projected onto the epidermal collar from which the feather arises (Lillie and Juhn, 1932, p. 132; Juhn and Fraps, 1936; Fraps and Juhn, 1936). Relying on the principles of localization and specificity, we can accordingly reconstruct the distribution of breast and saddle ectoderm in the germs of the chimaerae.

Figures 1–4 illustrate these principles. Each represents the collar (specifically, the ramogenous, or barb-forming, zone), divided through the ventral triangle and spread on a plane surface. The rhachis occupies the center. The apex of the feather is derived from the dorsal half of the collar as a series of barbs formed simultaneously *in situ* (Lillie and Juhn, 1932; Lillie and Wang, 1941). The loci of origin of new barbs then move rapidly in a ventral direction (transitional period), until those of opposite sides meet in the ventral triangle from which all remaining barbs arise. This is represented by the broken arrow above the ventral halves of each side of the collar. The direction of the tangential movement of barbs is represented by an arrow within the collar, and its extent during the transitional period by the numbers 1–5. The formation of the apex of the feather and of the barbs of the transitional period occupies a relatively inconsiderable time in regeneration of a feather, and these barbs therefore form only a small fraction of the vane. All later-formed barbs arise on the right or left side of the ventral triangle and by tangential movement pass during their growth completely across one lateral half of the collar to the rhachis. In so doing they acquire cells of the collar at each level, which contribute to their basal growth. The letter *M* on the extreme right and left indicates that most of the margin of the feather forms in this position.

Figure 1, *A* represents normal development, Figure 1, *B*, the development of chimaerae derived from union of lateral halves of breast (cross-lined) and saddle (stippled) papillae. Figure 2, *A* and *B* represent B/S combinations, Figure 3, *A* and *B* the S/B combinations, and Figure 4 twin chimaerae from an S/B combination. In Figures 2, *A* and 3, *A*,

representing bilaterally symmetrical chimaerae, the rhachis is centered in the dorsal component; in Figures 2, *B* and 3, *B*, representing bilaterally asymmetrical chimaerae, its position is excentric with reference to the dorsal component. Any degree of asymmetry may be so represented. The B and S ectoderm is represented in equal amounts in these diagrams; the possibility of unequal representation in certain cases would not affect the principles of the diagrams.

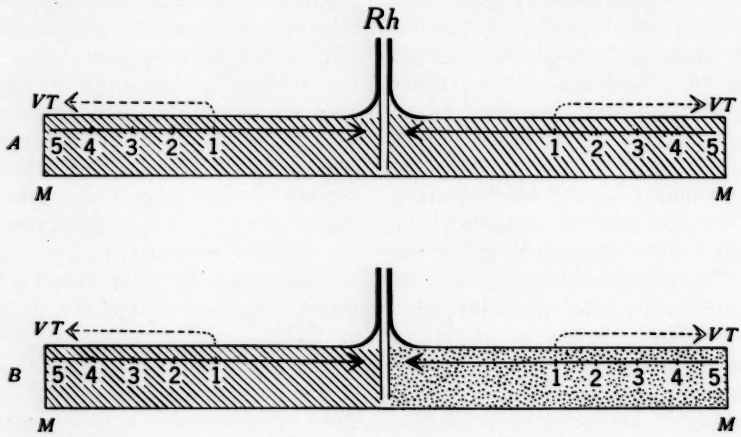

FIG. 1.—*A*, Collar diagram of the development of a normal breast feather. *B*, Collar diagram of the development of a bilateral chimaera from combination of right and left halves of a breast and a saddle papilla (Lillie and Wang, 1941).

Longitudinal chimaerae result only from B/S combinations (Fig. 2). The breast component occupies a dorsal position in the collar. B/S compositions are stable, i.e., the disposition of the components in the collar remains the same throughout the development of the feather. If through induction by the dermal papilla the rhachis forms in the center of the breast component, the saddle component will be equally distributed on its two sides. The result will be a feather with a B apex, followed by a series of barbs on each side, the marginal halves of which will be formed by saddle ectoderm and the central halves by breast ectoderm—in short, a symmetrical longitudinal (marginal) chimaera, such as is shown in Plate II, Figure 8. Four of the cases in Table 3 are of this type. If, however, the rhachis forms excentrically in the breast component (Fig. 2, *B*), one side of the chimaera will have a narrow, and the other a wide, saddle margin. In extreme cases a pseudobilateral type of feather will result (Pl. IV, Fig. 11).

In addition to the marginal chimaerae included in Table 3 there are four others (Table 1, dagger[†]), in which most of the feather is derived from the breast component, but the saddle component is represented by a narrow stripe on one or on both sides. There was thus very unequal representation of the components in the collar. These cases border on the normal breast feathers derived from B/S combinations (Table 2), in which the ventral (S) component failed to take or degen-

erated entirely. A less extreme example of reduction of the S margin is included in Table 3 (89:9).

The fluff length in marginal chimaerae is the same, or nearly so, on the two sides of the feather, and the ratio ranges from 2.4 to 2.9 (8 cases, Table 3) in contrast to 2 for the normal breast and 3 for the normal saddle; it thus appears to be influenced by both components. Total length is also influenced by both components and is intermediate between breast length and saddle length (cf. Table 3).

The growth rate of marginal chimaerae approximates that of breast feathers, which is more rapid than that of the saddle (Table 6); it is thus determined by the dorsal component. Even in the extremely asymmetrical cases, classified as pseudobilateral (B/S), this is also the case (Table 6).

The marginal chimaera furnishes a crucial test both of tangential movement and also of accretion of cells from the ramogenous zone of the collar as a regular contribution to the growing bases of the barbs. If barbs grew only by multiplication and differentiation of their own cells, they would maintain throughout their entire length the composition that they received at the time of origin at the sides of the ventral triangle. The existence of saddle and breast segments in the individual barbs in correspondence with the distribution of saddle and breast cells in the collar proves both the theory of tangential movement and the theory of accretion of cells from the collar during tangential movement and growth in a more direct way than by analysis of the development of normal feathers (cf. Lillie and Juhn, 1938, p. 440).

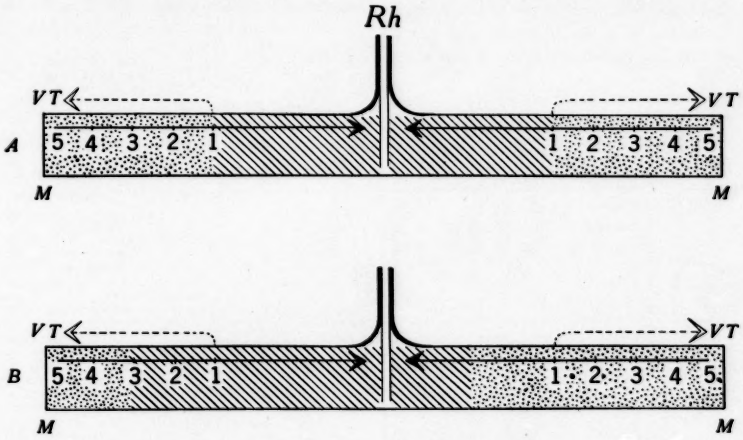

FIG. 2.—Collar diagrams of the development of longitudinal (marginal) chimaerae, B/S combinations. *A*, Symmetrical combination. *B*, Asymmetrical combination.

Transverse chimaerae, i.e., those with a saddle apex and breast base, result only from S/B combinations (Fig. 3, *A* and *B*). The saddle component occupies a dorsal position in the collar, and the breast component ventral positions on both sides. Such a composition of the collar is not stable throughout development, but the breast cells gradually invade the saddle area and eventually com-

pletely replace it. The direction of displacement is the same as that of the tangential movement of the barbs, and the B/S combinations show that it does not occur in the reverse direction (see discussion, p. 17). Symmetrical and asymmetrical locations of the rhachis may be distinguished (Fig. 3, *A* and B).

Transverse chimaerae always possess a saddle apex, followed by a series of intermediate barbs with breast margins of gradually increasing extent (Pl. III, Figs. 9 and 10) and saddle centers correspondingly diminishing until the entire length of barbs is breast, and the remainder of the feather also. Asymmetrical examples lead over in extreme cases to the pseudobilateral type.

The fluff ratio of symmetrical transverse chimaerae is the same, or nearly so, on the two sides and ranges from 2.1 to 2.9 (cf. Table 4). The total length is intermediate between breast length and saddle length.

The growth rate of transverse chimaerae is the normal saddle rate during the first part of its growth, corresponding to the saddle composition of the apical part of the vane; the rate then increases

TABLE 3

THE LONGITUDINAL (MARGINAL) CHIMAERAE

(Measurements of marginal chimaerae)

Total length, fluff length, and fluff ratio of the marginal chimaerae individually. The average length of normal breast feathers of the region concerned is about 100 mm., and of the saddle feathers 150 mm. Numbers 89:9, 91:7, 301:8, and 302:10 are symmetrical, the remainder more or less asymmetrical.

Number	Type	Generations	Total Length (Mm.)	Fluff Length (Mm.)	Ratio T/F
89:8*.....	B/S	First	120	42	2.9
		Second	127	47	2.7
		Third	129	45	2.9
89:9.....	B/S	First	135	48	2.8
		Second	137	50	2.7
		Third	137	50	2.7
91:7†.....	B/S	First	97	38	2.6
		Second	96	39	2.5
301:8.....	B/S	First	118	42	2.8
		Second	133	49	2.7
		Third	140	50	2.8
302:10..... (died)	B/S	First	115	47	2.4
303:8*.....	B/S	First	118	49	2.4
		Second	128	53	2.4
303:9*.....	B/S	First	122	49	2.5
		Second	122	49	2.5
303:10*....	B/S	First	110	45	2.4
		Second	125	48	2.6

* Asymmetrical.

† This feather is exceptionally short for a marginal chimaera (both generations). The bird was in bad health and died after the completion of the second generation of this feather.

TABLE 4

THE TRANSVERSE CHIMAERAE

(Measurements of transverse chimaerae)

Total length, fluff length, fluff ratio, and length of the apical saddle component of the transverse chimaerae individually. In the case of the extremely asymmetrical feathers 91:4 and 304:1, fluff length and fluff ratio are given separately for each side.

Number	Type	Generations	Total Length (Mm.)	Fluff Length (Mm.)	Ratio T/F	Length of S Portion (Mm.)
89:1.....	S/B	First	122	49	2.5	58
		Second	127	50	2.5	63
		Third	129	54	2.4	66
91:4..... (asymmetrical)	S/B	First	Defective; apex missing			
		Second	124	B:58 S:45	2.1 2.8	
301:3.....	S/B	First	115	40	2.9	52
		Second	120	42	2.9	58
		Third	124	43	2.9	61
301:2.....	S/B	First	101	42	2.4	41
		Second	117	45	2.6	53
		Third	117	45	2.6	60
304:1..... (asymmetrical)	S/B	First	139	B:57 S:51	2.4 2.7	51
		Second	145	B:63 S:60	2.3 2.4	60
		Third	147	B:65 S:61	2.3 2.4	60
304:5.....	S/B	First	117	55	2.1	50
		Second	122	57	2.1	57
		Third	119	61	2.0	50

to normal breast rate, corresponding to the breast composition of the remainder of the feather (Table 6). The distinction is clearly marked.

union of lateral halves of breast and saddle papillae (Lillie and Wang, 1941). Pseudobilateral chimaerae derived from S/B combinations differ in important re-

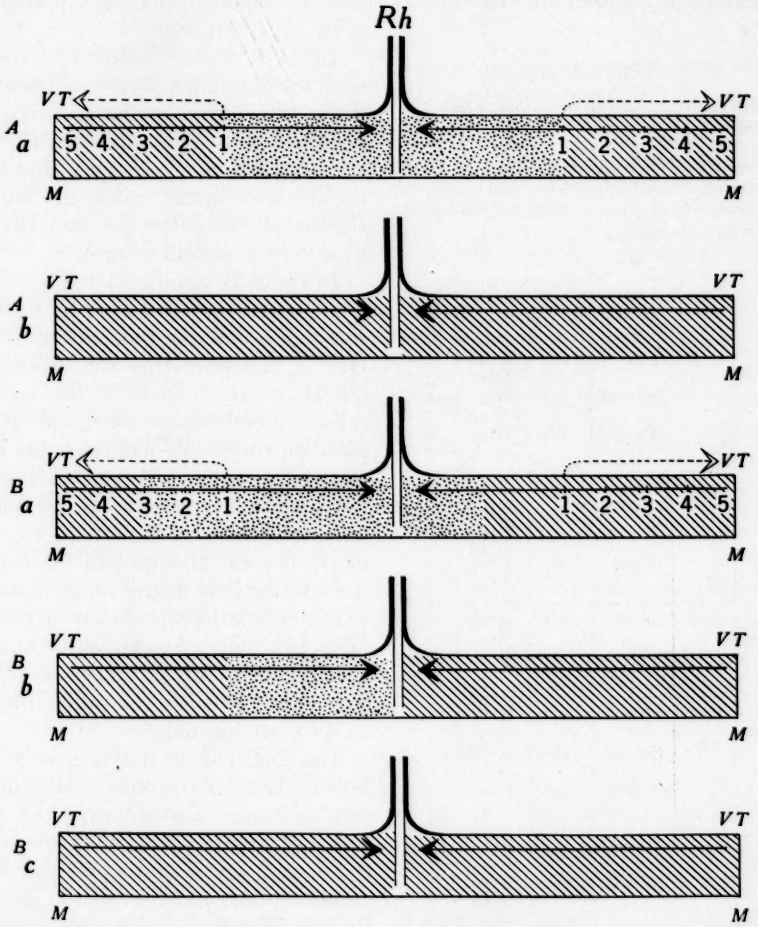

FIG. 3.—Collar diagrams of the development of transverse chimaerae, S/B combinations. *A*, Symmetrical combination: *a* and *b*, stages to show replacement of S by B cells (see text). *B*, Asymmetrical combination: *a*, *b*, and *c*, stages to show replacement of S by B cells (see text).

The *pseudobilateral chimaerae* are so named because, although one side is predominantly breast and the other saddle, this is not a clear-cut condition as it is in the true bilateral chimaerae derived from

spects from those derived from B/S combinations (Pl. IV, Figs. 11 and 12). It will be noted in these figures, for instance, that the web on the saddle-like side is much broader in the B/S combination

than in the S/B combination and that, moreover, in the former, breast characters extend at the apex onto the saddle-like side and, in the latter, saddle characters extend at the apex onto the breast-like side.

TABLE 5*

THE PSEUDOBILATERAL CHIMAERAE
(Measurements of pseudobilateral chimaerae)

Total length, fluff length, and fluff ratio of the pseudobilateral chimaerae individually. The last two measurements are given separately for the breast-like and saddle-like sides.

Number	Type	Generations	Total Length (Mm.)	Fluff Length (Mm.)	Ratio T/F
90:1.....	S/B	First	120	B:48	2.5
				S:40	3.0
		Second	118	B:52	2.3
				S:48	2.5
		Third	123	B:55	2.2
				S:44	2.8
90:2.....	S/B	First	123	B:46	2.7
				S:31	4.0
		Second	126	B:56	2.3
				S:40	3.2
		Third	127	B:57	2.2
				S:42	3.0
90:3.....	S/B	Second	114	B:58	2.0
				S:45	2.5
		Third	120	B:60	2.0
				S:45	2.7
90:9.....	B/S	First	148	B:65	2.3
				S:40	3.7
		Second	147	B:68	2.2
				S:41	3.6
		Third	146	B:68	2.2
				S:41	3.6
304:7.....	B/S	First	115	B:50	2.3
				S:45	2.6
		Second	127	B:56	2.3
				S:50	2.5
304:8.....	B/S	First	113	B:55	2.1
				S:45	2.5
		Second	130	B:65	2.0
				S:50	2.6
304:9.....	B/S	First	106	B:50	2.1
				S:54	2.0
		Second	114	B:58	2.0
				S:59	1.9

* Since this table was prepared, an additional generation has appeared in all these feathers except 90:9. The measurements in each case are substantially the same as in the preceding generation.

Figures 2, *B* and 3, *B* illustrate the compositions from which the two kinds of pseudobilateral chimaerae are derived; but the excentricity of the rhachis in the dorsal component may be supposed to be even more extreme.

In the B/S combination in accordance with the diagram a portion of the apex on the saddle side will be breast; and, as the composition is stable, the breast cells will extend all along the rhachis, thus widening the web on the saddle-like side. On the breast side below the apex there will be a narrow saddle margin.

In the S/B combination, on the other hand, a portion of the apex on the breast side will be saddle; but, as the composition is unstable, this will be replaced quickly on the breast-like side by breast cells. Though replacement will also take place on the saddle-like side, this will be relatively slow; it will proceed from margin to center and will not be attained until the region of the fluff, if the asymmetry is great enough (Pl. IV, Fig. 11). An intermediate degree of asymmetry in S/B combinations is shown in Plate III (Fig. 10), with a longer saddle apex and appearance of breast characters at the base of the vane on the saddle-like side, as is to be expected.

The length of the fluff is greater on the breast than on the saddle side in both combinations—also an expected result. The fluff proportion approximates that of the normal breast on its own side in S/B combinations, and that of the saddle on its side (Table 5). In B/S combinations the fluff ratio is in the same sense but less regular. Total lengths of both types are intermediate. The growth rate of pseudobilaterals of B/S derivation approximates the normal breast rate and that of S/B derivation the normal saddle rate (Table 6).

Twin chimaerae will be produced when a rhachis is induced both in the dorsal

and in the ventral components. The conditions under which this may happen have been discussed already (p. 7). Figure 4 represents the theoretical composition of the collar in such cases. The position of the rhachis in the ventral component (in this case B) is bound to be excentric, as already explained, though in the dorsal component it may be either central or excentric. The location of the two ventral triangles (*VT*) related to the two dorsal fields is secondarily determined by the extent of control exerted by each field on the available material to right and left. The collar is arbitrarily divided in the diagram along one of the ventral triangles. The diagram will apply in principle to all four cases (Table 1). The expectations based on the diagram will be that one partner (on the left in Fig. 4) will have an S apex, but the right side will be S, and the left side will have the characters of a transverse chimaera; the partner on the right will have a B apex, the right side will be B, and the left side will have the characters of a marginal chimaera. The asymmetry of each partner will be a mirrored image of the other, except for the dominance of B cells, when in a ventral position, over S cells. The four pairs of twins actually produced (Table 1) correspond very closely to the theoretical requirements (cf. Pl. V, Fig. 13).

III. SUCCESSIVE GENERATIONS OF CHIMAERAE

About 75 per cent of chimaerae emerge from the mouth of the follicle between 15 and 21 days after the operation—an average of 18 days. Compared with the time of emergence of normal breast (8–10 days) and saddle (10–12 days) feathers after plucking, there is a delay of about 1 week. In some cases, however, the delay is about 3 weeks. After the first generation the later ones also appear to be somewhat delayed in emergence, but much less than the first. In general, the physiological state of the papillae seems only rarely to be entirely normal.

The numbers of generations obtained in each case at the time of writing are recorded in Tables 3, 4, and 5. Total length shows a tendency to increase slightly in each generation, owing, no doubt, to improvement in regulation of the papilla with time; but the fluff ratio is quite constant. More striking than the data shown in these tables is the fact that the successive generations, including twins, always appear in the same classes (enumerated in Table 1) and that even

TABLE 6

PER DIEM INCREMENTS OF CHIMAERA FEATHERS

Per diem increments of feather-chimaerae. These are calculated only for the straight part of the total curves of growth. They are made on second- or third-generation feathers. Measurements were made every third day, although only the total increments are recorded, except in the case of the transverse chimaerae, in which the total increments are divided into two successive periods in which per diem increments are different.

For comparison it should be stated that in the portions of breast and saddle tracts used normal growth rates are 2.3 mm. per diem for the breast and 1.85 mm. for the saddle.

Feather	Generation Measured	Type	Days	Total Increments (Mm.)	Per Diem Increments (Mm.)
301:8 (B/S)..	Third	Longitudinal	32	65	2.00
89:9 (B/S)..	Third	Longitudinal	32	62	1.94
301:3 (S/B)..	Second	Transverse	28	49 (S part)	1.75
			22	47 (B part)	2.14
304:5 (S/B)..	Second	Transverse	28	51 (S part)	1.82
			22	53.5 (B part)	2.43
304:7 (B/S)..	Third	Pseudobilateral	32	66	2.06
304:8 (B/S)..	Third	Pseudobilateral	32	66	2.06
304:9 (B/S)..	Third	Pseudobilateral	32	78.5	2.45
90:1 (S/B)..	Third	Pseudobilateral	32	58	1.81
90:2 (S/B)..	Third	Pseudobilateral	32	53.5	1.68
90:3 (S/B)..	Third	Pseudobilateral	32	60	1.87

certain individual peculiarities tend to recur. It should be remarked also that successive generations of chimaerae show more individual variability than those of normal feathers.

There can be no doubt, therefore, that the composition of the *papilla* established after healing is quite stable as regards both epidermal and dermal constituents. This result agrees with the repetition in successive generations of the types of defective feathers and of twins produced by operations on normal papillae (Lillie and Wang, 1941). It contrasts with the instability of the *collar* in S/B combinations, which is a function of development and not of the organization of the germ. It is true that the collar receives its mosaic composition from the papilla; but after it is once formed it is an independent system. Changes that occur in the collar in the course of regeneration of the feather have no effect upon the original composition of the papilla.

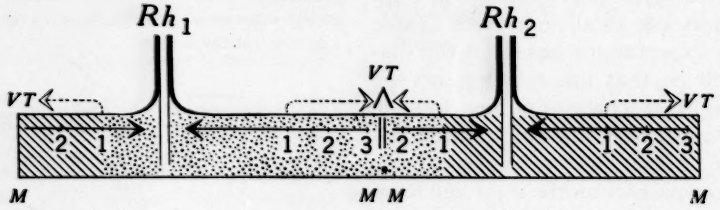

FIG. 4.—Collar diagram to show development of twin chimaerae from an S/B combination (see text)

IV. DISCUSSION

1. THE CHARACTERS OF THE FEATHER AS A WHOLE

The lengths attained by feather-chimaerae are intermediate between those of the normal components (Tables 3, 4, and 5). As the first generation is likely to reflect the immediate effects of the operation, the measurements of the second or third generation are to be preferred for comparison. In most cases they are near the mean between the breast (100 mm.) and saddle (150 mm.), but in two cases (301:1, Table 4; and 90:9, Table 5) the lengths are closer to the saddle. There is some variation besides, but the tendency to approximate the mean is unmistakable. As the B and S components should be quantitatively equal on the average, this suggests that each component plays an equal part in the determination of length. If this is so, the deviants from the mean should show preponderance of one or the other component. This is difficult to confirm, however.

The fluff ratio in the normal breast feather is about 2 and in the normal saddle about 3. In the strictly bilateral chimaera described in our previous paper, produced by union of lateral halves in which one side is all B and the other all S, the fluff ratio is 2 on the B side and 3 on the S side, in spite of the fact that the length of the feather is intermediate; and hence the fluff is absolutely longer on the B side and shorter on the S side than in the normals. Fluff ratio does not appear to be affected by length in this case. We may therefore carry this principle forward to the D/V chimaerae now under consideration.

In pseudobilaterals of D/V derivation, such as shown in Plate IV (Figs. 11 and 12), the fluff is longer on the breast-like side. In Table 5, which records all the pseudobilateral feathers, it will be seen that the fluff is longer on the breast-like

side, except in 304:9, and that the fluff ratios on the breast-like side in the later generations are above the normal breast ratio in most cases and on the saddle-like side frequently below the normal saddle ratio. The length of the fluff thus records very consistently the preponderance of B and S cells on the two sides. The ratio of fluff to total length is not so consistent. But there can be no doubt that each component plays a part in the determination of these characters in chimaerae of D/V derivation.

This conclusion is supported by longitudinal (marginal) chimaerae (Table 3), in which length of fluff is approximately the same on both sides, so that only one figure is given, and fluff ratio is intermediate between the normals, though sometimes approaching that of the saddle.

In transverse chimaerae (Table 4) the fluff ratio is more variable. But in this case it is difficult to see how the S component can play a part, inasmuch as the fluff is derived exclusively from the B component. The ratio given is the usual one of total length of the feather divided by length of fluff. If it were related to the length of the B component alone, the figures would be still more variable, owing to difference in length of this component in the individual feathers. But in the asymmetrical cases (91:4 and 304:1) the fluff is longer on the side with more B, and the ratios are correspondingly affected.

Feathers of different tracts are characterized by different and specific *rates of growth*, referable to differential rates in the respective collars. The specific rates are the same throughout the entire extent of the collar, thus maintaining the elongation of the feather cyclinder in a straight line. Do the different levels of the collar from dorsal to ventral act independently or is there a pace-setter to which all other parts conform?

Feather-chimaerae should furnish an answer to this question, because dorsal and ventral portions of the collar possess different specific rates of growth. If these persisted, then in B/S combinations, for instance, the feather cyclinder would be curved ventrally, owing to more rapid elongation of its dorsal half. But this never occurs. There is, therefore, some principle that regulates the discrepancy. This might conceivably be some kind of quantitative balancing of the two components, or it might be that one component or the other sets the pace.

In Table 6 the rates of growth per diem of 5 B/S chimaerae are recorded. These are to be compared with a normal rate of about 2.3 mm. per diem for corresponding breast feathers and 1.86 for saddle feathers. The mean would be 2.08. Only 1 of the B/S chimaerae equals the mean and, indeed, it exceeds the normal breast rate. On the other hand, none possesses a growth rate as low as that of the normal saddle.

The S/B combinations (Table 6) must be considered in two groups. The 2 transverse chimaerae exhibit during their saddle period a rate of growth below that of the normal saddle, and during their breast period 1 exhibits a rate slightly above the mean and the other a rate above that of the normal breast. The 3 pseudobilaterals of S/B origin possess a uniform rate of growth approximating that of the normal saddle.

Comparison with normals would be based on the assumption that no ill effects on rate of growth follow the operation and that the physiological condition of combined papillae is as good as that of normal papillae. But this appears to be unjustified by the results, which show that rates are not only pretty consistent-

ly below this comparison but are also quite variable, as might be anticipated, owing to variation in degree of persistent injury of the operated papillae.

This suggests that the rates of growth of B/S and S/B chimaerae should be compared with one another rather than with normal values. B/S combinations possess a significantly higher rate of growth than any of the S/B combinations, except in the B part of transverse chimaerae. If rate of growth of feather-chimaerae were determined by quantitative balance of B and S cells, which are originally equal in amount, this should not be the case.

The results are consistent only with one assumption, viz., that the dorsal component, and more especially the rhachis, is pace-setter for the rate of growth of the entire feather cylinder. In all B/S combinations, including pseudobilaterals, the rhachis is derived from the fast B cells (Fig. 2, *A* and *B*), and their rate of growth is greater than that of S/B pseudobilaterals, in which the rhachis is derived from S cells (Fig. 3, *B*), as far as the vane is concerned at least. The pseudobilaterals furnish the argument for confining pace-setting to the rhachis and not to the entire dorsal component of the collar, because only a narrow strip of B or S cells, as the case may be, is found on one side of the rhachis. Where the composition of the rhachis changes in transverse chimaerae, the rate also changes.

Twin chimaerae present a special problem in this connection, for here there are two rhachi proceeding from a single collar and growing within a single feather cylinder. The development of the partners may begin at the same time, in which case the lengths will be the same (Pl. V, Fig. 13), or they may begin at different times, in which case the partner of later origin will be relatively short. The rhachis of one partner is of S origin and the other of B origin; the latter will maintain this composition throughout development, but the former will change to B, as in transverse chimaerae generally. If there is any conflict in rates of growth between the two partners, according to the principle that the rhachis acts as pace-setter, it will be of brief duration only, and the effect is not likely to be detectable.

In normal development typical growth rates of breast and saddle feathers are not established until after origin of the rhachis on the ninth or tenth day of regeneration, before which the rates are the same for both kinds of feathers, much lower than the typical rates of either (Lillie and Juhn, 1932, pp. 134–35). This is quite in agreement with the hypothesis that the rhachis acts as pace-setter in the growth of the feather.

Of the characters of the feather as a whole (p. 2), total length, fluff ratio, and form of the feather are intermediates in the chimaerae and appear to be determined roughly by quantitative proportions of the components. Each of these characters is specific in its own tract. How the necessary adjustments between the components are made is difficult to imagine, but it is certain that they occur in the collar before any segregation of characters of specific kinds occurs. These adjustments are probably by transmission of stimuli rather than by transportation of substances; such functions in the collar are presumably operative in tract-specific development also. Rate of growth, on the other hand, is determined by the specific B or S composition of the rhachis, acting as a pace-setter for the entire collar. The change of rate of growth of the entire feather cylinder in transverse chimaerae, when the rhachis changes from S to B, is particularly il-

luminating. Pace-setting also implies transmissive action.

2. PARTS OF THE FEATHER

The development of parts of the chimaerae is dominated by the specific potencies of B and S cells, but in the line of contact of the two kinds of cells important reciprocal effects are to be observed.

The instability of S/B combinations in the collar already described was an entirely unexpected result. We had anticipated that both B/S and S/B combinations would be stable after they were once established and that two types of marginal chimaerae would arise, viz., breast in the center and saddle at the margins in B/S and saddle in the center and breast at the margins in S/B. The appearance of the transverse type of chimaera in the second case was at first a complete puzzle. We have, indeed, breast at the margins and saddle in the center in individual barbs of the transitional zone of transverse chimaerae (Pl. III, Figs. 9 and 10), but the length of the marginal breast component rapidly increases in successive barbs at the expense of the central saddle component, until the latter is entirely replaced. This indicates alteration of the cellular composition of the collar through gradual replacement of the dorsal S by the ventral B cells, such as is represented in Figure 3, *A* and *B*. But, if so, does not the reverse also occur in B/S combinations?

We considered the possibility that the marginal chimaerae might be interpreted as transverse chimaerae, reciprocals of S/B. The fact that the marginal chimaerae always have a breast apex made a start on such an interpretation, but we looked in vain for a transitional zone, and the base was certainly not all saddle. Measurements of growth rates were then undertaken, which show (Table 6) that the marginals exhibit a constant rate approaching that of the breast feathers throughout, whereas the transverse chimaerae start out with a saddle rate and change at the transition zone to the breast rate. On all counts the marginals appear as a separate class from transverse chimaerae.

We have, then, the apparently anomalous situation that S cells cannot replace B cells in the collar but that B can replace S, though in one direction only, viz., from ventral to dorsal. If replacement were to take place also in the opposite direction, the B/S combination would yield a chimaera feather with B apex, followed by a series of barbs with B center and S margin of diminishing width until the entire feather became B. Such a type of chimaera does not occur.

There are two factors in replacement of S by B cells: In the first place, since S cells do not replace B cells whatever their position, there must be some intrinsic factor of the B cells. The B cells in the male and in the capon possess a higher rate of growth than S cells, as exhibited in the faster growth of breast than of saddle feathers. There are also other tract-specific characters of B and S cells than rate of growth, none of which seems so likely to be involved in the present phenomenon. In the second place, position is also a factor. The main condition in the factor of position likely to be effective is the direction of tangential movement, also present in S/B combinations in the same direction. Neither the intrinsic factor nor the direction of tangential movement is effective alone in replacement of S cells by B cells.

The tangential movement brings B cells into competition with S cells in S/B combinations and S cells into competition with B cells in B/S combinations. The difference between marginal chimae-

rae derived from B/S combinations and transverse chimaerae derived from S/B combinations therefore demonstrates that, wherever such competition exists, the B cells dominate, owing to intrinsic properties, whether of higher growth rate or of some other kind.

Substitution of brown for black pigmentation always occurs to some extent in the B component of marginal (B/S) chimaerae, but not in transverse (S/B) chimaerae. In this substitution certain regularities are evident. In the first place, the tip and a short narrowing continuation along the rhachis of B/S chimaerae, whether true marginals or pseudobilaterals, is always black. This part is derived from B cells exclusively as represented in Figure 2, and its black color corresponds to expectation. In a good many cases all the remainder of the B component is brown. In other cases the black color extends the entire length of the rhachis from the tip as a stripe, either on one or on both sides, which varies in width in different feathers; in intermediate cases it is interrupted in the middle of the vane. The width of the longitudinal black stripe is roughly inversely proportional to that of the S margin. As an extreme example, the four cases starred in Table 1 are entirely black, except for the very narrow marginal S stripes. It is to be specially emphasized that black never occurs at the margin and brown never appears in the center of the B component. Successive generations of the same feather may vary in the amount of the central black stripe; thus the second and third generations of the feather shown in Figure 8 (Pl. II) had no central black stripe.

The general statement is that in barbs possessing an apical S segment and a basal B segment there is a tendency for substitution of brown for black color in the adjacent part of the B segment. Moreover, the extent of this effect bears a relation roughly proportional to the length of the S segment.

We have seen that S cells cannot replace B cells in the collar; they do, however, affect the pigmentation of B cells in the direction of tangential movement and not in the reverse direction. The mechanism of development of the feather requires that S components, in a ventral position in the collar, pick up B cells in B/S combinations when they reach them in the course of tangential movements and that they use them in their growth, seeing that they cannot replace them (Fig. 2). They are thus in contact with the latter in a formative stage; but, as the barbs continue to grow, their bases become farther removed from S cells, which become progressively keratinized; hence any influence that they may have on B cells decreases toward the rhachis.

The effect of this influence is substitution of brown for black in the B component. The black color is due to melanin formed in chromatophores, which do not develop in the S component. It is generally agreed that melanin is due to oxidation of tyrosine through the intervention of an oxidase and that a brown stage is passed through before the final black color is attained. Although the nature of the effect exerted by the S cells is unknown, it could be formally explained by assuming that the S cells contain a substance which inhibits the oxidizing process in the B melanophores and brings it to a halt in the brown stage. Such an effect would naturally decrease with distance.

In breast feathers of Brown Leghorn capons two or more years old partial substitution of brown for black is not uncommon. The brown areas of such feathers are in the form of transverse

bands which are usually more or less incomplete. They thus indicate temporary disturbances during growth which inhibit the oxidation of the melanin precursor. The pattern of the brown is thus entirely different in these cases from that of marginal chimaerae. Nevertheless, in addition to the effect of S cells in marginal chimaerae, there is frequently a "dilution" of black in the breast component of all classes of chimaerae due to a sprinkling of small brown areas, which must be attributed to some general cause.

Apart from these reciprocal influences, viz., the replacement of S by B cells when the latter are in a ventral position and the limited substitution of brown for black pigment in the melanophores of the B component when S is ventral, the B and the S components appear to differentiate entirely independently. The widening of the web that occurs on the S side of pseudobilaterals of B/S origin (Pl. III, Fig. 12) is to be attributed to the presence of B cells on that side; half of the marginals recorded in Table 3 are asymmetrical, and all degrees of width of the web are to be found in them, proportional to the amounts of the B components on the two sides. Reciprocal influence of B and S components would seem to be excluded as far as development of barbules is concerned.

3. CONCLUSION

In these experiments components with definitely different tract specificities are brought together. They have in common the character of genetic restriction to feather development, but they differ in respect to the kind of feathers produced. As the combinations are autoplastic, there are no constitutional antagonisms to be encountered. But adjustments of the innate developmental tendencies of the components are obviously involved in the attainment of the individualized development exhibited by the chimaerae. Within this harmonious result we find both self-expression and reciprocal interaction of the components.

The analysis of feather-chimaerae fits very neatly into the framework of the mechanism of development as hitherto presented in this series of papers and is not at any point in conflict. It reinforces some principles previously stated, e.g., that of the special conditions of development of the apical portion of the feather and also that of the tangential movements of the barbs and of the accretion of cells to their growing bases from the successive regions of the collar. The analysis also confirms the principles of induction by the dermal papilla, of the prospective significance of parts of the papilla, and of the specificity of B and S epidermal cells as presented by Lillie and Wang (1941).

In the development of the feather there is a uniquely sharp transition between a period of correlative differentiation, in which characters of the feather as a whole are determined, and a following period of self-differentiation of the parts. This corresponds to an earlier conception of embryonic development in general, which is now outmoded, owing to discovery of the persistence of integrative actions throughout the life of the organism. But the feather is a part possessing neither vascular nor nervous connections, composed, in fact, of dead cells, and cut off from such influences after it is formed; hence the sharp transition between a period of integration and one of self-differentiation in its development.

V. SUMMARY

Autoplastic combinations of dorsal and ventral halves of breast (B) and

PLATE I

FIG. 5.—Normal breast feather.

FIG. 6.—Normal saddle feather.

FIG. 7.—Bilateral chimaera from L/R combination (Lillie and Wang, 1941).

PLATE II

FIG. 8.—Symmetrical longitudinal (marginal) chimaera, B/S combination (301, No. 8, Table 1); first generation.

PLATE III

FIG. 9.—Symmetrical transverse chimaera, S/B combination (89, No. 1, Table 1); first generation.

FIG. 10.—Asymmetrical transverse chimaera, S/B combination (304, No. 1, Table 1); first generation.

PLATE IV

FIG. 11.—Pseudobilateral chimaera, S/B combination (90, No. 2, Table 1); second generation.

FIG. 12.—Pseudobilateral chimaera, B/S combination (304, No. 8, Table 1); first generation.

PLATE V

FIG. 13.—Twin chimaerae, S/B combination (89, No. 3, Table 1); second generation

PLATE I

PLATE II

PLATE III

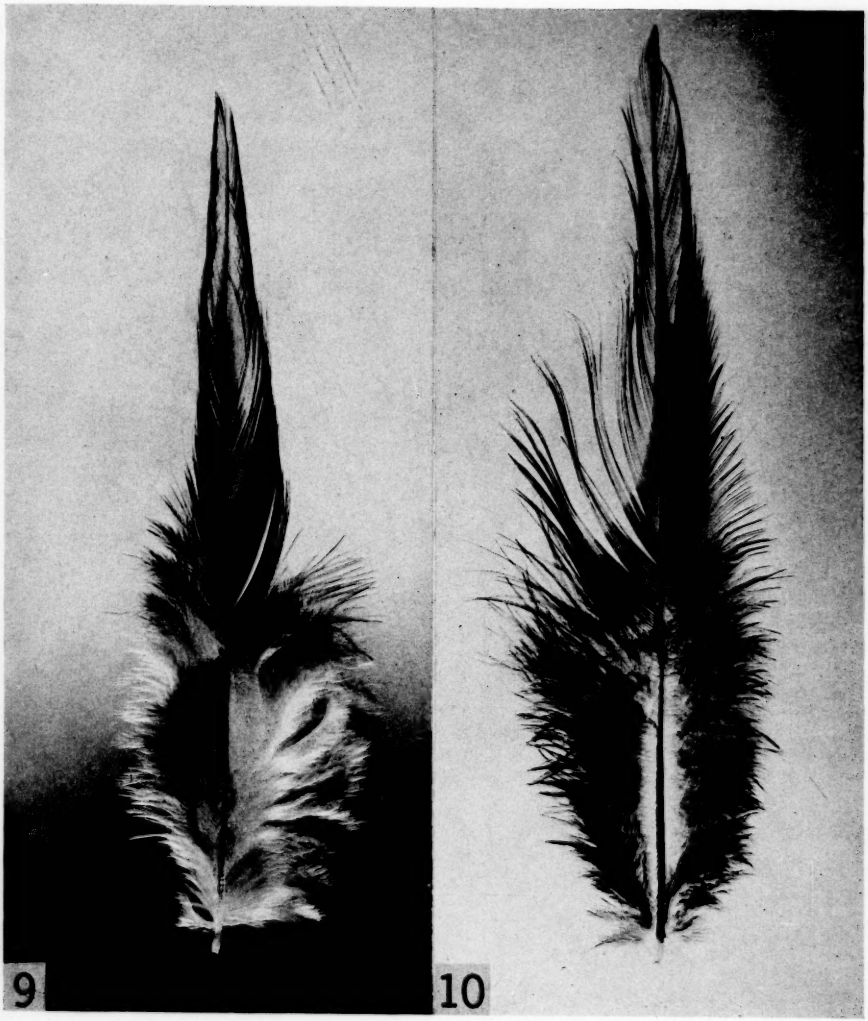

PLATE IV

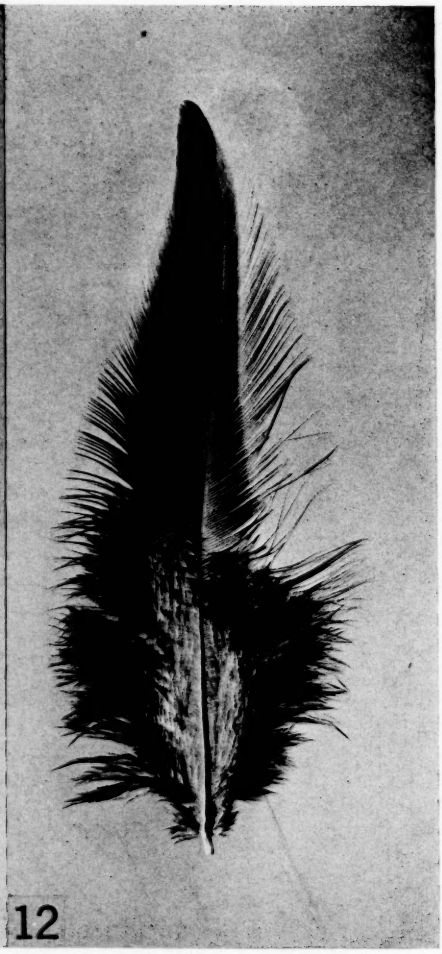

PLATE V

saddle (S) papillae of regenerating feathers of Brown Leghorn capons were made in the follicle of one of the components (host component). From such unions feather-chimaerae exhibiting characters of both tracts develop and are succeeded by similar feathers in successive regenerations.

Symmetrical B/S combinations produce longitudinal ("marginal") chimaerae, B in the center and S at the margins.

Symmetrical S/B combinations produce transverse chimaerae, in which the apex of the feather is S and the base B with a transitional zone between, composed serially of barbs with increasing B apexes and diminishing S bases.

Asymmetrical combinations of both sorts produce asymmetrical chimaerae of their respective types leading to pseudobilateral chimaerae of two kinds.

Occasionally a single combination produces twin feathers, each of which is a chimaera.

Theoretical analysis shows that the B/S composition of the collar is stable throughout development. Hence apexes of barbs form from ventral S cells and bases from the dorsal B cells. The S/B composition of the collar is, on the other hand, unstable, the ventral B cells replacing the dorsal S cells gradually but completely in the direction of tangential movements of the barbs.

Although S cells cannot replace B cells in the collar, they nevertheless act on them in the direction of tangential movement and inhibit the oxidation of the melanin precursor, so that B melanophores adjacent to S cells remain brown and never reach the black stage.

The lengths and the fluff ratios of feather-chimaerae are intermediate in relation to B and S feathers The rates of growth are determined by the dorsal component, in which the rhachis seems to act as pace-setter for both components.

All the results fit into the theory of the mechanism of feather development as stated in previous papers of the series.

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THE HEART-FORMING AREAS OF THE EARLY CHICK BLASTODERM

(Six text figures; one plate)

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IN AN earlier series of experiments (Rawles, 1936) it was demonstrated, by testing the developmental potencies of small measured pieces of the early chick blastoderm (head-process stage) on the chorio-allantoic membrane, that particular organ tissues develop from rather definite regions; that is to say, organ-forming areas are localized to a remarkable extent.

So characteristic are some organ areas of particular levels that it was found possible to designate along the anteroposterior axis an eye-forming level, a thyroid-forming level, a mesonephros-forming level, etc. In addition to this relation to anteroposterior axiation many organs were found to show a definite relation to mediolateral organization. Heart and liver, for example, were found to develop in lateral areas only; other organ tissues, like chorda and suprarenal, in median areas only, while many—like eye, ear, and mesonephros—developed in both median and lateral areas but with a higher-frequency percentage in the median region. While the above-mentioned experiments were concerned primarily with locating in a general way the position of the various areas with organ-forming potencies in the blastoderm as a whole, the intention from the beginning was to follow this analysis further and determine rather exactly, as far as the method allows, the extent and boundary of each of the areas already designated. So far the eye-forming area is the only one which has been so analyzed (Clarke, 1936). In the present investigation an attempt has been made to study in much the same way the heart-forming areas, i.e., to locate in the early blastoderm the specific region which is capable of producing cardiac muscle when grafted to the chorio-allantoic membrane and to determine as far as possible whether this capacity varies in intensity within the boundary of the specifically located area or field.

[1] The author wishes to acknowledge her indebtedness to Professor B. H. Willier for his interest and advice during the course of the experimentation, to Mr. John Spurbeck for the excellent photomicrography, and to Mrs. H. D. Stalker for the diagrammatic drawings.

EXPERIMENTAL METHOD

The method used for determining the position, extent, and shape of the area in the blastoderm which has the capacity to produce cardiac muscle in chorio-allantoic grafts consists of dividing the blastoderm into a number of small pieces by the use of longitudinal and transverse cuts appropriately placed at carefully measured distances from the primitive pit and then transplanting each of these isolated pieces to the chorio-allantoic membrane of host embryos of 8–9 days' incubation. Here the transplant was allowed to grow for 8 or 9 days, after which time it was removed and examined histologically for the occurrence of cardiac muscle.

Glass needles with very finely drawn-

out tips at an angle of 45° were used for making the cuts. The various pieces were transplanted to the host membrane with capillary pipettes of suitable size. In this series many of the blastoderms were kept in chicken-blood serum instead of saline (Locke's) solution during the cutting and transplantation procedure. Since the method employed in cutting and handling small pieces of the early blastoderm has been described somewhat in detail elsewhere (Rawles, 1936), it will not be considered further here.

THE DONORS

Most of the embryos had head-processes ranging in length from bare visibility to the formation of a definite head fold. A few definitive primitive-streak blastoderms were also used. In the presomite stages the blastoderm is a thin sheetlike area which, with the exception of an anterior region—the proamnion—consists of three germ layers—ectoderm, mesoderm, and endoderm—without any visible indication of heart primordia. The pericardial region of the coelom (amniocardiac vesicles) begins to appear with the formation of the first 1 or 2 somites; and even by the time the head fold is formed there is some indication of a delamination of the lateral mesoderm anterior to the node. The first indication of the heart proper is a thickening of the splanchnic mesoderm of the pericardial coelom to form the myocardium, which occurs at approximately the 3-somite stage.

EXPERIMENTS DETERMINING THE LOCATION OF THE HEART-FORMING AREAS

A. THE ANTEROPOSTERIOR EXTENT

In the attempt to locate the area with heart-forming potencies it has been necessary to employ a wide variety of isolations. Two series, represented by Figures 1 and 2, in which the blastoderm was divided into 11 and 18 pieces, respectively, have furnished very useful data pertaining to the location of the heart areas, as well as other organ-forming areas. These data, in fact, were the basis for the general maps already presented in some detail. Although not designed to limit precisely any of the areas, they do throw considerable light on their position and extent in the blastoderm as a whole. Therefore, we shall consider them here with as much brevity as possible and with particular reference to the heart.

Series 1.—Figure 1 shows how the blastoderm was divided. A transverse cut made anywhere from 0.1 to 0.2 mm. anterior to the pit separates an anterior portion designated as Level I, which includes a good portion of the head-process and the anteriormost parts of the area pellucida. Another cut, similarly made but posterior to the pit, separates a second level containing the pit and node field—Level II. A third transverse cut, placed 0.4–0.5 mm. posterior to the pit, cuts off a post-nodal strip designated as Level III and separates it from a posterior portion containing approximately the posterior half of the streak and the hindmost part of the blastoderm—Level IV. This last region was further cut into right and left halves by placing a cut down the middle of the primitive groove. At the other three levels two longitudinal cuts were made, 0.1–0.2 mm. to each side of the pit, giving a median strip 0.2–0.4 mm. wide. Thus at Levels I, II, and III we have left and right lateral portions and median portions. Grafts were recovered from all 11 pieces. Median pieces at all levels produced more grafts and larger ones than either lateral or posterior pieces. Grafts were obtained

less frequently from right pieces than from left and were as a rule smaller. Altogether, a few over 200 grafts were obtained, about one-half of which have been examined histologically. Structures were identified by their cytological and histological constitution. Cardiac muscle was identified in both right and left lateral grafts from Levels I, II, and III. It was not found in any grafts of Level IV and with one exception was absent entirely from the grafts of the median pieces at all levels. The exceptional case came from a median piece of maximum width, taken 0.12 mm. posterior to pit (Level III) at a stage rather earlier than medium head-process. The heart appearing was somewhat aberrant. While we are concerned primarily with heart tissue, it should be clearly understood that, in all these grafts, heart is not the only structure present but is associated with many others more or less characteristic of the level. Two usually quite intimately associated with heart are liver and thyroid. Thyroid is more or less restricted to Level I, where it occurs in median as well as lateral pieces. Only rarely does it show up in Level II. Liver, on the other hand, occurs at all the levels where heart appears, although less frequently posterior to the node—Level III. More often than not, the two tissues are in actual continuity.

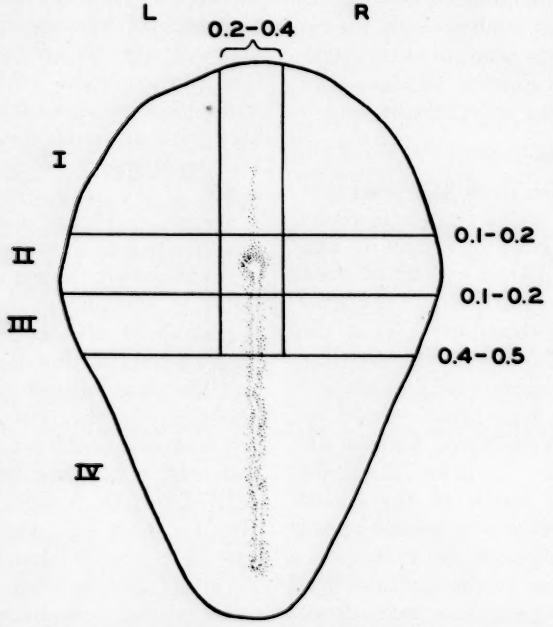

FIG. 1.—Diagram of head-process blastoderm, showing the location of the various cuts; Series 1. Numerals I–IV indicate the levels which, with the exception of IV, consist of a median and two lateral pieces. L and R = left and right sides, respectively. Measurements in millimeters. × 28.

Of the two, heart develops much more frequently in grafts, so is not uncommonly found without liver development. It very rarely happens, however, that liver occurs in the absence of heart tissue. Although heart can be obtained from a

wide region lateral to the pit (Levels I, II, and III), it should be pointed out that the frequency with which it appears in these grafts varies considerably at the different levels. For example, lateral grafts at the node level (II) show the highest frequency, 90 per cent. Anterior to this, in Level I, the frequency decreases to about 71 per cent, while posteriorly in Level III there is a still greater drop—to 52 per cent. There is no very noticeable difference between the percentages from the right and left sides, which is definitely the case with the development of many other organs, such as eye and kidney.

Just how far the areas with heart potency extend anteriorly in Level I or posteriorly in Level III the present series does not tell us. For clarifying this point other experiments were necessary. Before turning to them let us in summary point out that the present data do contribute several facts worthy of emphasis. First, it is clear that the areas which can form heart tissue are located approximately 0.2 mm. to each side of the primitive pit; second, they extend over a considerable area of the blastoderm—both anterior to and posterior to the node level, the posterior limits somewhere between 0.2 and 0.4 mm.; and, third, they show a gradient in the frequency with which they produce heart in the grafts at the various levels along the anteroposterior axis, the highest frequency percentage occurring at the node level.

Series 2.—In these experiments two more transverse cuts were made than in the preceding series, and the two longitudinal ones carried to the posterior margin of the area pellucida, thus dividing the blastoderm into 18 pieces. Figure 2 illustrates the manner of cutting. If it is compared with the preceding diagram (Fig. 1), it will be seen that Level A is equivalent to the anteriormost portion of Level I. A is always made anterior to the tip of the head-process, regardless of the length of process, which means that it varies considerably in its distance from the pit, i.e., it is nearer to the pit when the process is short than when it is long. Level B always includes the tip of the process. Its posterior boundary varies from 0.26 to 0.4 mm. from the pit and is therefore usually about 0.1 mm. more anterior to the pit than the posterior boundary of Level I. In a general way A and B, then, correspond to Level I of the former series. It will be further observed that in this series a transverse cut passes through the primitive pit, dividing the node region into an anterior part, Level C, and a posterior part, Level D. These two correspond in a general way to the nodal strip—Level II—of the preceding series. Level C includes, then, the anterior portion of Level II and approximately 0.1 mm. of the posterior part of Level I; and, similarly, Level D includes the posterior part of Level II and the anterior part of Level III. The E strip is equivalent to the posterior portion of Level III plus the anterior portion of Level IV. The remaining posterior area of the blastoderm is included in strip F. The two longitudinal cuts made 0.1–0.18 mm. to either side of the pit extend the full length of the blastoderm and give us a median piece at each of the six levels. It is readily seen that this series with the additional transverse divisions would be expected to furnish more definite information as regards the anteroposterior extent of the heart-forming areas.

Some 250 grafts have been examined histologically. As observed in the former series, so here, the capacity of the transplanted pieces to survive and grow under graft conditions, as well as the amount of tissue produced, exhibit conspicuous dif-

ferences along the anteroposterior and the mediolateral axes. The anterior and posterior ends of the area pellucida yield grafts much less frequently than do those portions which include either the node or the head-process, and they are invariably smaller. At every level except A, which is quite anterior to the axis and exceed-

median pieces containing heart muscle, 1 came from Level C, the other 2 from D. In all, the strip was at its maximum width. Two came from a donor in the early head-process stage, the third from a donor with a medium head-process.

Heart is a rather common constituent of the lateral grafts at Levels B, C, and

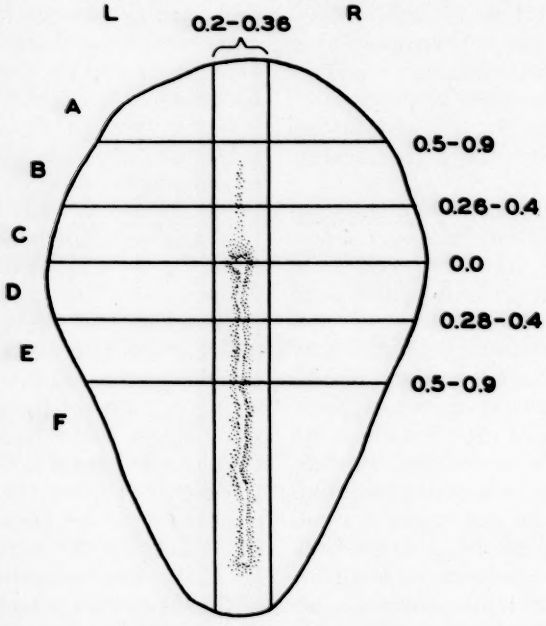

FIG. 2.—Diagram of head-process blastoderm, showing the location of the various cuts; Series 2. Letters A–F indicate the six transverse strips, each of which is separated into a median and two lateral portions. L and R = left and right sides, respectively. Measurements in millimeters. × 28.

ingly difficult to obtain grafts from at all, the median pieces show the greatest power to produce grafts. This capacity is much reduced in the left pieces and still more so in the right pieces. With rare exception (3 cases out of 106) heart does not occur in grafts of the median pieces, which is in agreement with the preceding results and to be expected, since the median strip is about the same width in both series. Of these 3 exceptional

D. It occurs infrequently at Level E and is entirely absent from Levels A and F. It is associated with various other organ-forming areas characteristic of the particular levels. Again the most intimate association is with liver and thyroid. Thyroid is a characteristic component of all the portions of strip B. It seems definitely associated with the tip of the head-process along with the eye and forebrain. It extends further anteriorly to the tip

than any of the other areas. In 3 cases it has developed in pieces cut 0.1–0.18 mm. anterior to the process tip.

The capacity to produce liver very nearly coincides with the heart region. It does not extend so far posteriorly, i.e., it has not been observed in Level E (0.3–0.4 mm. posterior to pit). The frequency with which it appears in grafts at all levels is definitely lower than that of heart. Heart occurs in 71 per cent of the grafts from Level B, 81 per cent from Level C, reaches a high of 90 per cent at Level D (posterior half of node level), then drops off abruptly to 21 per cent at Level E. The heart development in Level E is represented in 4 grafts, 2 left and 2 right, by small pieces of rather typical cardiac muscle. The feeble development here contrasts with the excellent growth exhibited at the other three levels. The further fact that these 4 positive cases occurred when the transverse cut separating Levels D and E was made 0.3 mm. posterior to the pit and never occurred when the cut was made farther behind the pit indicates strongly that we are dealing with the posteriormost portion of the areas. This was confirmed by other experiments which will be described subsequently.

To summarize: the data of this series show rather convincingly that the parts of the blastoderm capable of producing heart are situated 0.18–0.2 mm. to either side of the mid-line (primitive pit) and extend anteriorly not over 0.5 mm. from the pit and posteriorly not over 0.4 mm. from the pit. Further, the capacity to form heart is more intense at the level of the posterior half of the node and grades off from this region more gradually and over a longer distance anteriorly than posteriorly.

Series 3.—It has been clearly demonstrated in the analysis of various levels of the blastoderm that the portion well anterior to the head-process shows weak growth and very slight capacity for differentiation in grafts. The only structures which have appeared in this anteriormost region, other than the primordial germ cells which persist and undergo modification, are nonspecific epithelial tubes and pearls and stratified epithelia, possibly epidermis. Similarly, the region containing the posterior half of the streak, the lateral portion in particular, is greatly restricted in its capacity for growth and differentiation. While the lateral pieces give the same type of epithelial structures as all pieces of the anterior portion, Level A, the middle pieces frequently give a more highly organized epithelial tube with muscular walls, which is unquestionably posterior digestive tube. Also skin may occasionally develop. Especially is this the case when the cut separating Level F is made farther posteriorly to the pit than 0.6 mm. Also, experience has shown that at these extremities of the area pellucida, the size of the piece is definitely a factor in the realization of its developmental potencies; that is to say, grafts can be obtained more frequently and the differentiation of the characteristic structures is more complete when the strips are taken as a whole rather than divided into median and lateral pieces.

In view of the above-mentioned facts it seemed important to analyze further these anterior and posterior portions as wholes in order to be certain that heart would not occur in them even rarely. Therefore, experiments were undertaken in which both of these regions were progressively increased in their extent toward the primitive pit, that is, Level A was made to include varying amounts of the anterior portion of Level B and, similarly, Level F to include varying

amounts of the posterior portion of Level E (see Fig. 3).

Level A undivided.—Fifteen grafts have been examined in which the cut represented by the line *aa'* (Fig. 3) ranged from 0.2 mm. above the apparent tip of the process to bare contact with the tip. In none of these cases was carness. Heart developed in one-third of these, in only 1 case, however, when as little as 0.04 mm. of the process was taken and in 1 other when 0.06 mm. was included. In the other 4 cases approximately 0.1 mm. of the process tip was included and would be expected to give heart on the basis of our findings in Se-

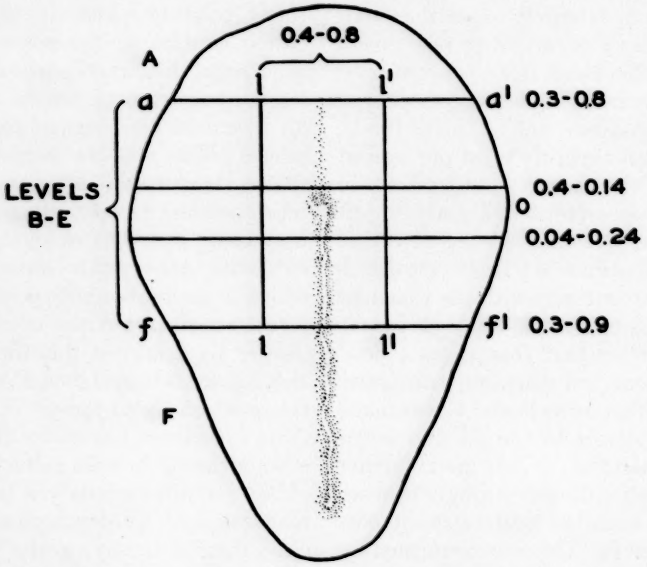

FIG. 3.—Diagram of head-process blastoderm, showing the location of the cuts; Series 3 and 4. Letters A–F indicate the various levels. The two longitudinal cuts *ll* and *l'l'* separate levels B–E into a median and two lateral portions. The transverse cuts *aa'* and *ff'* separate the undivided levels A and F, respectively, from the other divided levels. Measurements in millimeters. × 28.

diac muscle observed. Two grafts contained thyroid (0.08 mm. and 0.14 mm. anterior to the process tip), and one contained central nervous tissue in a whorl-like arrangement. Other than these, the structures present were the characteristic epithelial structures and germ cells already mentioned.

An additional number of grafts—18—has been examined, in which *aa'* (Fig. 3) was moved still further posteriorly to include from 0.04 to 0.2 mm. of the proc-ries 2. Therefore, if we include as much as 0.1 mm. of the process tip, we are well into the anterior end of the heart-forming area. Occurring with heart in these grafts are liver, thyroid, eye, forebrain, etc., which have been described before as characteristic of the strip including the process tip (Level B of Series 2).

From the above analysis, therefore, we may conclude that the anterior limit of the heart-forming areas follows very closely (0.04 mm. behind) the tip of the

process but definitely not extending beyond the tip. Since the length of the process varies in the different phases of head-process development, the distance of the anterior limits of the areas from the pit will vary accordingly. In a medium head-process stage with a distinct rodlike process, the distance from the process tip to the pit will be approximately 0.6 mm., and the anteriormost limit of the heart-forming area will be approximately 0.56 mm anterior to the pit.

Level F undivided.—Thirty-four grafts of this type have been examined histologically. In 24 of these the transverse cut *ff'*, separating Level F from the rest of the blastoderm, varied from 0.3 to 0.6 mm. posterior to the pit and in 10 from 0.6 to 0.9 mm. posterior. From this rather large group only 3 grafts developed heart, in which cases the dividing cut *ff'* was 0.3, 0.38, and 0.4 mm., respectively, from the pit. It will be recalled that in Series 2, heart developed in 4 cases in Level E (2 left; 2 right) when the anterior boundary of the piece was 0.3 mm. from the pit, but never when the boundary was moved farther posteriorly. The fact that 2 cases have been obtained farther posteriorly in the present series is extremely interesting and may be interpreted as the result of grafting larger undivided pieces of this region of the blastoderm, where growth and differentiation are decidedly inferior. There can be no doubt from the infrequent occurrence of heart that we are here dealing with the posteriormost limit of the heart-forming area.

As regards other structures occurring in grafts of these posterior undivided pieces, gut with smooth muscle coats—undoubtedly intestine, skin, feathers, cartilage, and bone—are the principal constituents. Grafts from pieces as far posterior as 0.38 mm. contain, in addition to the above, mesonephros and ganglia. One graft 0.3 mm. from the pit contained unorganized central nervous system tissue in the form of whorls of cells. It is very rare that any central nervous system tissue develops in grafts taken this far posteriorly. Beyond 0.6 mm., gut with muscle and occasional small pieces of cartilage are the only recognizable structures. There are many nondescript epithelial tubules, which are characteristic of grafts of any peripheral region of the blastoderm where growth and differentiation are poor. One cannot help being impressed by the gradual falling-off in growth and differentiation of structures as the cuts are made progressively farther posteriorly.

The foregoing analysis of the posterior undivided pieces of blastoderm has demonstrated that the areas possessing heart potency extend at least as far as 0.4 mm. posterior to the level of the primitive pit.

B. THE MEDIOLATERAL EXTENT OF THE HEART-FORMING AREAS

Series 4.—So far the experiments described have been concerned primarily with the anteroposterior extent of the areas, yet the failure of cardiac muscle in median grafts of both the 11- and the 18-piece series (Figs. 1 and 2) has contributed also one important fact relative to their mediolateral location, namely, that they lie on each side of the blastoderm, beginning approximately 0.2 mm. from a median line passing through the primitive pit. In only 4 cases out of 145 median grafts examined histologically was there even the slightest trace of cardiac development. Such findings give us, therefore, a good starting-point for the present series of experiments to determine the extent of the two areas mediolaterally.

Figure 3, Levels B–E, will illustrate the method of sectioning, as well as the variations in distances from the pit. Both transverse and longitudinal cuts were again employed, although the latter are more important at this time. Often one or more of the transverse divisions was omitted in order to throw together two levels and increase the size of the very small lateral pieces. Especially was this done when the median strip reached its maximum width of 0.8 mm.

Starting at a 0.2-mm. distance from the primitive pit—the maximum width of the median strip of Series 1 and 2—the longitudinal divisions *ll* and *l'l'* (Fig. 3) were made progressively farther from the pit, approaching the area pellucida margin, until a median strip 0.8 mm. (0.4 mm. to each side of the pit) had been tested. At its maximum width such a strip is exactly twice as wide as that of Series 1 and 2, heretofore described.

It was thought that by gradually increasing the width of the median strip it might be possible to include all the region with heart-forming capacity in the median grafts and finally exclude it altogether from the laterals. Unfortunately, it was not so simple a matter, for as long as any grafts could be obtained from the steadily decreasing lateral pieces they still showed a good development of cardiac muscle.

The great difficulty here was to obtain grafts from the lateral pieces, particularly when the separating cut was between 0.3 and 0.4 mm. to each side of the midline. When it is recalled that the entire width of the head-process blastoderm at its widest point—node level—is only approximately 2 mm. and that, owing to its pearlike shape, the width decreases rapidly both anteriorly and posteriorly, especially posteriorly, the smallness of the left and right pieces can be appreciated. Furthermore, it is a well-established fact that there is a decided decline in the capacity of portions of the blastoderm to develop into grafts as the distance from the node field and head-process is increased either anteriorly, posteriorly, or laterally, i.e., peripheral pieces show a striking inferiority in developmental capacity. It was not, therefore, practicable in the present series of experiments to increase the width of the median strip beyond 0.8 mm. For further analysis of the far-lateral areas another type of experiment was designed and will be described subsequently.

A total of 94 grafts was examined histologically—45 median, 28 left, 21 right. According to expectation, the frequency with which heart appeared in the median grafts increased with the width. For example, when *ll* and *l'l'* were made 0.2–0.3 mm. to each side of the pit, heart occurred in 1 case out of 9, but in 7 cases out of 28 when cut 0.3–0.4 mm. to each side of pit. When the width of the median piece was increased to 0.4 mm. or slightly more, 3 out of 9 grafts showed heart. Grafts were obtained from the median pieces with a rather high frequency—60 per cent. They showed good growth, often several millimeters in diameter. Central nervous tissue usually formed the bulk of the graft, although other structures characteristic of the various levels occurred also.

The lateral grafts were very much smaller than the median and could be very easily overlooked without careful examination of the membrane (with binocular dissecting microscope) at the transplantation site. Usually they consisted of a small vesicle filled with a colorless fluid with a tiny pinkish mass of tissue either at the surface or a little deeper. In one-fourth of the left and nearly one-third of the right grafts this pinkish mass

showed pulsation at the time of graft removal. Where there was no pulsation, histological examination revealed cardiac muscle in the great majority of cases—i.e., in 24 of the 28 left grafts and in 16 of the 21 right grafts.

There was no noticeable falling-off in the heart frequency of the lateral pieces as the width of the median piece increased. If the grafted piece showed any growth at all, the chances were that heart would occur. In the 4 cases in which grafts were recovered from lateral pieces when the median strip reached its maximum (0.4 mm. to each side of pit) heart developed in 3 (1 right; 2 left). Liver is associated with heart in approximately one-fourth of these grafts, also thyroid at the appropriate level. Other more generalized structures, such as gut, muscle, cartilage, skin, etc., show up rather frequently.

In conclusion, this series of experiments has demonstrated clearly that the heart-forming areas extend from 0.2 mm. to each side of the pit (median line) to a region at least 0.4 mm. distant. As far as the data from the various transverse levels go, they strengthen the evidence already presented relative to the anterior and posterior limits.

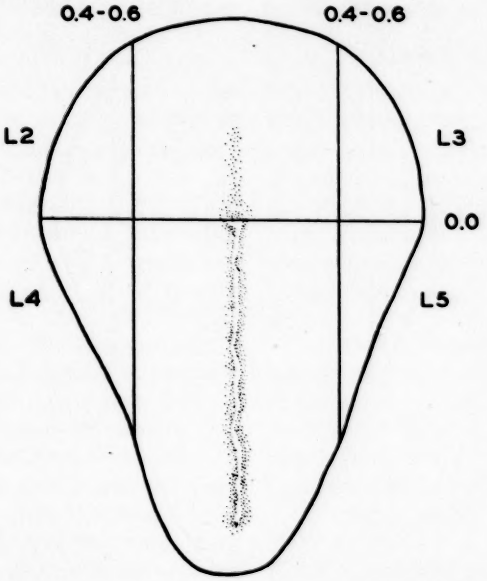

FIG. 4.—Diagram of head-process blastoderm, showing the location of the various cuts; Series 5*a*. Letters L_2–L_5 indicate the portions transplanted. Measurements in millimeters. × 28.

Series 5.—To test the far-lateral portions (0.4 mm. or more from mid-line), where the size of the grafted piece is an important, even a limiting, factor in growth and differentiation, experiments were next attempted in which the transverse divisions were either reduced to one passing through the blastoderm at the pit level or omitted altogether, thereby increasing the area of the lateral pieces. These will now be described in order.

a) One transverse cut through the blastoderm at the primitive pit.—Figure 4 illus-

trates the method of sectioning. As in the preceding series, longitudinal cuts were made on either side of the pit beginning at 0.4 mm. and gradually increasing to 0.6 mm. With the one transverse cut passing through the primitive pit, the lateral strips were thus divided into upper ($L2$, $L3$) and lower ($L4$, $L5$) portions. Often the lower pieces were considerably smaller than the upper, depending upon variations in the width of the posterior half of the blastoderm and the length of the process.

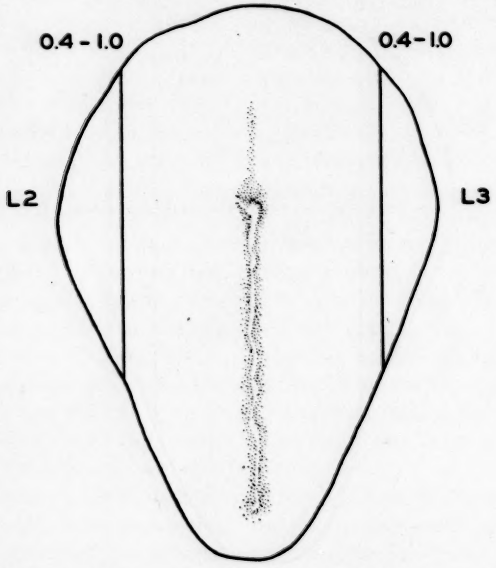

FIG. 5.—Diagram of head-process blastoderm, showing the location of the two longitudinal cuts. Letters $L2$ and $L3$ indicate the portions transplanted. Measurements in millimeters. × 28.

The membrane at the transplantation site was examined in 66 cases in which the host remained alive. In only 35 of these (13 from $L2$, 6 from $L3$, 7 from $L4$, 9 from $L5$) was there even the slightest evidence of a membrane reaction. These were examined histologically. Eighteen, approximately one-half, showed some differentiation (10 from $L2$, 4 from $L3$, 1 from $L4$, 3 from $L5$); and, of these, 13 contained small masses of typical cardiac muscle (7 from $L2$, 4 from $L3$, 1 each from $L4$ and $L5$). Pulsation was exhibited in 8 cases.

The grafts containing heart came from pieces taken between 0.4 mm. and 0.6 mm. lateral to the primitive pit. No pieces beyond 0.6 mm. showed any kind of differentiation. Also it should be noted that the anterior pieces $L2$ and $L3$ showed a greater capacity for differentiation than the smaller, posterior pieces $L4$ and $L5$. In view of the small number of grafts obtained, it seemed impracticable to continue subdividing these far-lateral areas. Therefore, this type of isolation was given up for that described in (*b*).

b) *No transverse cut.*—Figure 5 illustrates the method of cutting. Beginning 0.4 mm. to each side of the pit the two

longitudinal cuts separating pieces $L2$ and $L3$ from the rest of the blastoderm were made progressively nearer the junction of the area pellucida and the area opaca. To compensate for the gradual decrease in the size of the lateral pieces as the margin of the pellucid area was approached, portions of the area opaca were included.

In all the 116 cases in which the host remained alive the chorio-allantoic membrane was examined with magnification for any reaction which might have occurred at the graft site. Where there was even a faint suspicion of a thickening, histological examination was made. Of 48 so studied (26 from $L2$, 22 from $L3$), 41 showed some growth and differentiation, hence could be called grafts (24 from $L2$, 17 from $L3$). Cardiac muscle differentiated in 21 of these (14 from $L2$, 7 from $L3$); 8 from the left side and 1 from the right were pulsating at the time of removal from the host membrane.

In this series heart occurred as far laterally as grafts could be recovered. In only 5 cases were grafts obtained from pieces taken as far away as 0.8 mm. from the pit. Three of these contained only traces of heart tissue, 2 from right pieces, 1 from left. Beyond 0.8 mm. no grafts could be obtained. The frequency with which grafts were recovered and the percentage containing hearts were highest from pieces taken between 0.4 mm. and 0.7 mm. from the pit. Of 30 such grafts obtained, 18 (60 per cent) contained heart. Beyond 0.7 mm. only 9 grafts were recovered; 3 (33 per cent) of these showed cardiac muscle. Thus, there is a decided falling-off in both graft frequency and development of cardiac muscle as the limit of the area pellucida is approached.

The advantage gained in this series by transplanting the right and left pieces undivided is clear, when we consider that heart has been obtained 0.2 mm. farther from the pit, thus extending the limits of the heart-forming area considerably farther laterally than heretofore in series where the left and right pieces were subdivided. There is one disadvantage, however, in transplanting undivided pieces: there is no way of knowing whether the cells with heart-forming potency are scattered throughout the piece or more or less localized within some part of it.

Throughout this entire Series 5, in which we have been concerned with extreme lateral pieces of the blastoderm (0.4 mm. and beyond the primitive pit), the inferiority of the right side as compared with the left in its capacity to produce either grafts or hearts has been observed. For example, out of a total of 35 grafts recovered from left pieces, 23, or 66 per cent, contained heart, which exhibited pulsation in 13, or 56.5 per cent, of the cases; 12 (50 per cent) of the 24 grafts from right pieces contained heart muscle and only 4 (33 per cent) showed pulsation.

Structures other than heart which have appeared in these grafts are liver, tubules, probably gut, with and without smooth muscle coats, and large cells resembling primordial germ cells. In 11 cases (6 left; 5 right) liver developed either in contact with the heart or near by. It never occurred in grafts taken farther from the pit than 0.62 mm. In fact, the majority of the grafts containing this structure (8 of the 11) came from pieces cut around 0.4 mm. from the pit.

It seemed reasonable to expect that the area with heart-forming potency might extend to the limits of the area pellucida, since heart tissue has shown up as long as grafts could be obtained. Also it is well known that this region of the blastoderm is concerned with the ori-

gin of the omphalomesenteric veins and on this basis might be expected to have the capacity to form heart. Therefore, various further attempts were made to test the potency of the outermost 0.1–0.2 mm., from which so far no grafts had been procured. It is possible to increase the total area of the grafted piece by including portions of either the opaque area or the pellucid area anterior or posterior to the limits of the heart area, i.e., regions known to be incapable of forming heart tissue in grafts. But, although this was done, no heart tissue appeared farther laterally than heretofore obtained.

Thinking that another culture medium might favor differentiation in the extreme lateral pieces, a series of *in vitro* cultures was prepared. Cuts were made as usual and the pieces transferred by pipette to plasma–embryonic-extract clots on cover slips, which were inverted over depression slides and the edges sealed with paraffin. (This method has been described and used successfully by Rudnick, 1938*a*, 1938*b;* Spratt, 1940, and others, for growing portions of early blastoderms). Control cultures of similar pieces cut 0.4–0.7 mm. lateral to the pit, including the region known to give heart muscle on the chorio-allantois, were run simultaneously. From a total of 50 cultures which appeared in good condition, 18–36 hours after explantation, pulsating rounded masses of cells (hearts) were observed in approximately half of the controls; but in not a single case was there pulsation or even an accumulated mass of cells resembling the hearts of the controls in any of the pieces cut 0.8 mm. or more from the primitive pit. As far as they go, these results are interesting in showing good agreement between the two methods.

Whether or not the failure of the cells in these outermost lateral portions may be attributed to their lack of heart-forming potency or whether they are incapable of realizing this potency under the particular experimental conditions where very small pieces are isolated cannot be decided.

Summary.—Portions of the blastoderm beyond 0.4 mm. from the mid-line showed a decided inferiority in developmental capacity. There was a decided falling-off in both graft frequency and development of cardiac muscle as the limit of the area pellucida was approached. Only when undivided pieces were taken could grafts be recovered from the region 0.6 mm. from the pit. Beyond 0.8 mm. no differentiation was obtained either on the chorio-allantoic membrane or within *in vitro* cultures. Since traces of heart muscle occurred as long as grafts could be obtained, the limits of the potency field could not be definitely established. It is possible that a weak heart potency extends all the way to the junction of the pellucid and opaque areas.

THE GRAFT HEART

The heart which develops in chorio-allantoic grafts has its own characteristics and is quite different in many repects from the normal bird heart. It is simply a discrete lump or mass of typical cardiac muscle, generally round or oval shaped, without any indication of the four primary chambers. It is located usually at or near the graft surface and almost invariably partially surrounded by a space (pericardial cavity) filled with a colorless, coagulable, lymphlike fluid. There is no pericardium; the muscle mass is imbedded in the loose mesenchymatous tissue of the graft. Usually there is only 1 muscle mass, but there may be 2 or even 3 in contact or proximity.

The size of the heart varies consider-

ably, depending to a large extent upon the size of the transplanted piece and the portion of the blastoderm from which it is taken. Of 228 graft hearts examined in section, the dimensions of the long axis ranged from 75 μ to 1,500 μ. In 37 cases (16 per cent) the longest dimension was between 1,000 μ and 1,500 μ; in 137 cases (60 per cent) between 500 μ and 1,000 μ; and in 54 cases (24 per cent) under 500 μ.

The largest masses differentiated in grafts of lateral pieces when the vertical cuts were made 0.15–0.3 mm. to each side of the pit, and the transverse cuts 0.2–0.4 mm. anterior and 0.1–0.3 mm. posterior to this point (Series 1, 2, and 4). This region, it will be recalled, includes the center of the heart areas (see Fig. 6). When the cuts are made toward the outer boundaries of these fields, the masses of cardiac muscle developing are, as a rule, smaller. For example, in Series 5, where the transplants were taken 0.4 mm. lateral to the pit and outward, the size of the heart ranged from less than 100 μ to nearly 1,000 μ in the longest dimension, but the average size was small —between 200 μ and 400 μ.

It should be pointed out, perhaps, that great variation exists both in size of graft and in heart mass in transplants taken from the same region of the blastoderm and having presumably the same developmental potencies, owing, no doubt, to incidental conditions, such as inadequate attachment, inadequate blood supply, etc.

Figures 7–12 (Pl. I) illustrate the microscopic structure of the graft heart. The musculature, it will be observed, is typical in showing the same degree of development exhibited by the normal 10-day chick heart. The muscle fibers are arranged in interlacing bundles or strands between which are narrow slitlike meshes. Striations of the myofibrils are clearly visible in many places. The nuclei are oval shaped and scattered in the network of fibers at various distances from one another. Mitoses are frequently found.

The differentiation of the cardiac musculature is remarkably uniform in the grafts, regardless of their origin in the blastoderm. What seem to be slight variations in the general appearance of the muscle in section can in most cases be attributed to the rather peculiar and unusual angles at which the sections were often cut, owing to the fact that no exact orientation was possible. However, two types or grades of heart development have been distinguished, namely, compact and diffuse (cf. Figs. 8 and 9), the chief difference lying in the density of the arrangement of the muscle fibers. In 153 cases (67 per cent) the heart belongs to the first type—compact, the muscle mass being quite dense and firm in appearance. Only 19, or approximately 8 per cent, showed the diffuse type of differentiation. In these the muscle fibers composing the mass are much less crowded together and are separated by patches of mesenchyme so that the whole presents a rather loose, scattered appearance (Fig. 9). This type of heart was more or less confined to grafts exhibiting weak growth and occurred at no particular level or region of the blastoderm. It probably represents a weak attempt at heart formation.

In 55 grafts (24 per cent) both the compact and the diffuse types of differentiation could be found in the same heart mass. The greater portion was always compact, but certain diffuse regions of varying intensity could be found here and there. Figure 7 illustrates the occurrence of the two types in the same section.

The hearts are extremely well vascu-

larized by the chorio-allantoic blood vessels, which can be easily traced in section. A large central blood-filled lumen, which formed so commonly in the hearts from whole-blastoderm transplants of the same age (Willier and Rawles, 1931), was the exception here (2 cases). Small lumens occurred in 44 cases (19 per cent), but the vast majority showed none whatsoever. When a lumen does occur, it is lined with an endothelium. Also the character of the muscle fibers about it changes, becoming longer and coarser and showing a concentric arrangement.

Pulsation at the time of removal from the membrane was a rather common phenomenon. Where it was not spontaneous, it could often be induced by submerging the graft in saline heated to approximately 40° C. Occasionally pricking with a fine needle point would start contractions. The heart has no circulation of its own. When the muscle mass contracts, blood is forced along the connecting vessels, the effect being that of squeezing a rubber tube filled with liquid. The rate is variable and of no consequence, for in opening the eggshell to expose the graft and membrane the large chorio-allantoic vessels are necessarily severed and blood is lost from the graft.

Small clumps of ganglionic tissue were found frequently close to the heart mass or in contact when the lateral pieces were taken not more than 0.36 mm. from the pit. None ever appeared farther laterally. Since pulsating hearts formed in the far-lateral grafts, there can be no correlation between pulsation and the presence of ganglionic cells.

DIAGRAM SHOWING THE LOCATION OF THE HEART-SPECIFIC AREAS AT THE HEAD-PROCESS STAGE

The results of the foregoing experiments are summarized and represented graphically in Figure 6 for a typical medium head-process stage (length of process, approximately 0.6 mm.). The two large, bilaterally situated, shaded areas which occupy a considerable portion of the blastoderm (each is approximately 1 mm. × 0.6$^+$ mm.) are specifically endowed with heart-forming potency and capable of differentiating typical cardiac tissue when divided into small pieces and transplanted to the chorio-allantois of older host embryos. Beginning approximately 0.2 mm. on each side of the midline through the primitive pit they extend laterally to a region just beyond 0.8 mm., almost to the junction of the pellucid and opaque areas, giving a total width on each side of about 0.6$^+$ mm. Anteriorly each area follows quite closely (approximately 0.04 mm. behind) the level of the tip of the head-process, never extending beyond it. Posteriorly these areas extend somewhere between 0.4 and 0.44 mm., making the total anteroposterior length about 1 mm.

From the beginning of head-process formation until the end (formation of head fold) the anterior limit of the heart areas remains fixed, as does also the tip of the process. As the node moves back, this distance increases, thus bringing about an elongation both of the areas and of the head-process. The posterior limit does not remain fixed but moves back posteriorly as the node retrogresses, always maintaining a constant relationship with the pit (approximately 0.4 mm. distance). In consequence of these facts the total anteroposterior extent of the areas is longer in a medium or late head-process stage than in an earlier one.

The intensity of the shading within the areas represents in a general way the frequency of occurrence of heart in the grafts as described in the preceding series of experiments. It will be noted that

the portion of each area or field in which the self-differentiation capacity of heart is most firmly established (the smaller oval-shaped region) is located not quite in the center at the level of the node and process base. Toward the periphery the grafts could be recovered from these far-lateral pieces, heart tissue was present. We have no way of knowing, therefore, whether heart extends farther than the limits indicated on the map (Fig. 6). The chances are that it does. It seems reason-

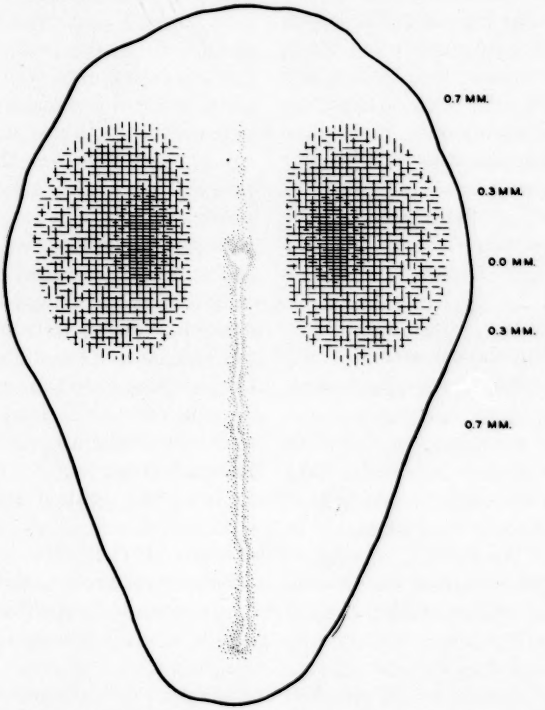

FIG. 6.—Map showing the location of the heart-specific areas in a head-process blastoderm. Depth of shading represents in a general way intensity or degree of developmental potency. × 35.

broken lines indicate that the capacity to produce heart is becoming weaker and weaker, finally fading out altogether.

Caution should be used in interpreting the outermost lateral boundaries of the areas. It will be recalled from Series 5*b* that it was very difficult to get grafts from lateral pieces taken beyond 0.6 mm. from the pit and that no grafts could be obtained from pieces cut farther than 0.8 mm. lateral to the pit. Yet, as long as able to suppose that mesoderm cells all the way out to the area opaca possess heart-forming potencies, since this region gives rise to the omphalomesenteric veins. In view of these facts the outermost lateral boundaries must be regarded as representing the limits of the capacity to produce grafts rather than the limits of the cells possessing heart-producing capacities.

The extraordinary size of the heart

areas can best be appreciated by comparing them with other organ-specific areas at this same stage. The eye-forming area, for example, which has been carefully mapped by Clarke, has a transverse diameter not exceeding 0.38 mm. and a longitudinal diameter of 0.15 mm. (see Clarke, 1936, Fig. 2). The mesonephric area is approximately 0.5 × 0.4 mm. at its widest point (see Willier and Rawles, 1935, Fig. 1). Both of these are quite small in comparison. Their position in the blastoderm affords a further contrast. While the heart is distinctly bilaterally located at this stage, both the eye and the mesonephric areas occupy median positions.

LOCALIZATION OF THE HEART AREAS IN PRESTREAK, EARLY-STREAK, AND DEFINITIVE PRIMITIVE-STREAK STAGES

The exact position of the heart-forming region in prestreak and early-streak blastoderms has not been definitely established, although there are many data bearing on it. Olivo (1928) found that pulsating heart muscle would develop in tissue cultures of the peripheral regions of unincubated blastoderms but not in similar cultures of the remaining central area (1 mm. in diameter). Using the same stage, Butler (1935) was able to show that heart muscle would develop rather frequently from both the anterior and the posterior halves when isolated and grown on the chorio-allantoic membrane. From these findings we cannot escape the conclusion that the capacity for heart formation is widespread in the prestreak blastoderm.

The most comprehensive studies on the localization of potencies in the early stages of the chick blastoderm are those of Rudnick (1938*a*, 1938*b*) and Spratt (1942). By culturing *in vitro* sections or areas of the blastoderm at various stages during the formation of the primitive streak, they have shown that the differentiation center of the heart area lies in the posterior half near the streak. Whether there are two separate areas bilaterally situated or one median area cannot be fully decided, since the median and lateral regions were not tested separately.

Rudnick (1932) presented a series of data obtained from chorio-allantoic grafts of measured strips of the anterior portion of the definitive-streak blastoderm which enables us to make some comparison regarding the location of the heart areas at this stage and the head-process stage. In her experiments a transverse cut was made across the area pellucida well behind the node and a longitudinal cut on each side of the node extending forward from the transverse cut as a base up to the area opaca. Thus the approximate anterior half of the blastoderm was divided into one median and two lateral strips. Heart developed from all three pieces—in 5 cases out of 11 from the right, 8 out of 12 from the left, and 8 out of 20 from the median pieces. If the data from the median strips are examined relative to the width of the middle strip, the evidence favors two bilaterally situated heart areas as in the head-process stage rather than one area which extends across the median region of the blastoderm. For example, when one or both of the longitudinal cuts were made 0.2 mm. from the primitive pit, heart appeared in 5 of the 8 grafts recovered; in 2 out of 4 grafts when one or both longitudinal cuts were made 0.18 mm. from the pit; and in only 1 out of 8 when the cuts were 0.11–0.18 mm. to either side of the pit.

It will be recalled from the data already presented for the head-process stage that heart showed up occasionally (4 out of 145 cases) in grafts of median pieces when the longitudinal cuts were

made 0.2 mm. from the pit. Such infrequent occurrence of heart tissue was interpreted as indicating that we were dealing with the periphery of the heart field where heart-forming potency is weak. It may be that the two heart areas are somewhat closer to the pit or mid-line at the definitive-streak stage than at the head-process stage, but the evidence from the data quoted seems against their extending across the mid-line as do both the eye- and kidney-forming areas at this stage.

Some data have been accumulated on the mediolateral extent of the heart areas at the definitive-streak stages, while experiments were being carried out on the head-process blastoderms. Cuts made like those in Series 5*b* (Fig. 5) have shown that heart can develop from lateral pieces taken as far away from the pit as 0.6 mm. Again the indication is that the lateral extent of the areas is approximately the same in the two stages.

A few data are also available which throw light on the anteroposterior extent of the areas. Hunt (1932) in a study of the potencies of various transverse levels of the primitive-streak blastoderm when grown on the chorio-allantoic membrane did not obtain heart from levels more posterior to the pit than 0.3 mm. No grafts from portions anterior to the node field (approximately 0.2 mm. from the pit) produced heart; but, if any part of the node field was included, then heart would develop.

My own data for this stage are in agreement. Heart has not occurred in grafts of regions more than 0.2 mm. anterior to the pit, but in one case a good development of pulsating heart muscle was obtained from the posterior undivided portion of a blastoderm taken 0.4 mm. behind the pit—0.1 mm. farther posteriorly than Hunt's data showed. Thus the anteroposterior length of the heart-forming area at this stage is approximately 0.6 mm.

As far as the data go, they seem to indicate that the heart areas are very similar in location and extent in the primitive-streak and the head-process stages, the chief difference being in the length anterior to the pit, which is greater in the latter stage owing to the formation of the process. It is quite probable that the areas specific for heart formation are always bilateral in the chick blastoderm, in view of their origin and intimate connection with the blood vascular system.

ORIGIN AND PROPERTIES OF THE HEART-SPECIFIC AREAS

By testing the self-differentiating capacity of various regions of the head-process blastoderm it has thus been possible to locate two rather large areas specifically endowed with heart-forming potency (see Fig. 6). While any part of these areas is capable of differentiating a typical cardiac musculature when isolated and grown independently on the chorio-allantoic membrane, the frequency with which heart is recovered varies considerably within the area. In other words, we find a gradient in developmental potency. There is a center where the differentiating capacity for heart is most firmly established within each area. This is represented in Figure 6 by darker shading and is located at the level of the node and base of the head-process, not exactly in the geometrical center. Grafts containing this part of the area produced heart muscle with the highest frequency. As the periphery of the area is approached, the developmental potency diminishes, as indicated by the decreasing frequency of heart occurrence in the grafts and represented in the diagram by less intense shading. Thus the heart-

specific areas appear to have all the characteristics of embryonic fields as described by various investigators for the amphibian and chick embryos. As already pointed out, heart differs from the other fields described for the chick at this stage (eye and mesonephros) in being entirely bilateral. In this respect it resembles more the limb field of amphibians.

Although the experiments have shown that these two relatively large areas possess heart-forming potency, we should not think of them necessarily as being absolutely fixed for heart production. Usually in embryonic fields the extent of the potency field is greater than the region from which the organ is actually differentiated in normal development and undergoes progressive restriction as development proceeds. This has been demonstrated for various organ fields. The amphibian-limb field as described by Harrison (1918) and others is a good example. The potency field—the region in which limb development can occur—is in earlier stages more extensive than the region of actual limb formation. The limb normally develops from the region where the potency is highest, nearer the anterior than the posterior border. Possibly in the heart, also, the region which gives cardiac muscle with the highest frequency is the portion which later forms the two primordia, but this has not yet been demonstrated.

From the present experiments we do not know whether the heart potency is restricted to the mesoderm only or whether the other germ layers play a part in the determination process, since the isolated pieces always contained all three layers. Evidence from *in vitro* experiments of Rudnick (1938*a*) and Olivo (1928), however, indicates that the endoderm is of minor importance in the definitive primitive-streak stage (approximately 16 hours' incubation). Both authors obtained functional heart muscle from mesectoderm only, but Rudnick states that similar isolations containing endoderm showed strikingly better heart differentiation, which is to be expected, since at least some of the lower mesodermal layer usually adheres to the endoderm. What effect the endoderm may have had on the origin of the heart potency prior to this stage is, of course, not known. Various experimental data have shown how very important the contiguous germ layers are in determining the origin and development of organ-forming regions (Adelmann, 1930; Holtfreter, 1938).

The distinct asymmetrical organization exhibited by organ-specific areas lying transverse to the anteroposterior axis—e.g., the eye (Clarke, 1936) and the mesonephros—does not show up in the bilaterally located heart areas. While the data do not indicate any striking differences in the shape or extent of the left and right areas, they do show that the developmental capacity of the left side of the blastoderm is superior to the right. It has been pointed out repeatedly in the preceding experimental section that grafts were obtained more frequently from left pieces than from the corresponding right pieces and that usually they were larger. The inferiority of the right side as compared with the left in its capacity to produce either grafts or hearts is most noticeable in Series 5, where extreme lateral pieces were grafted. Here, also, the hearts which developed in grafts from left pieces were pulsating in 13 out of 23 cases, as compared with 4 out of 12 from similar grafts of right pieces. Such obvious and consistent differences in the developmental capacity of the two sides of the blastoderm suggest a differential in

physiological condition from left to right.

Perhaps a word should be added in conclusion concerning the form which the heart takes in chorio-allantoic grafts. The fact that typical morphogenesis does not accompany the typical histogenesis observed is interpreted as the result of the abnormal mechanical conditions under which the graft develops rather than any restriction of the determination process. Kumé's experiments (1935) would seem to favor such an interpretation. He found that, even when the entire heart primordium or isolated pieces of it (8–20-somite chick embryos) were grown on the chorio-allantoic membrane, differentiation resulted still in the same masses of cardiac muscle without any indication of the usual four divisions. Such a result indicates the importance of extrinsic factors in bringing about a typical morphogenesis.

SUMMARY

1. In order to locate the regions of the early chick blastoderm (head-process stage) with heart-forming potency, the pellucid area was divided into a number of small measured pieces by means of a variety of transverse and longitudinal cuts appropriately placed with reference to the primitive pit. Each piece was transplanted separately to the chorio-allantoic membrane and allowed to differentiate for a period of 8–10 days, after which time the grafts were examined histologically for heart tissue.

2. The graft heart is a round or oval-shaped mass of histologically typical cardiac muscle without the usual four primary chambers, located at or near the graft surface and as a rule surrounded by a pericardial-like cavity. The size varies considerably (from 75 μ to 1,500 μ in length), depending to a large extent upon the size of the transplant and the portion of the blastoderm isolated. Pulsation occurred frequently.

3. Two large bilaterally situated areas specifically endowed with heart-forming potency have been located. Beginning approximately 0.2 mm. on each side of the mid-line (primitive pit), they extend laterally as far as grafts could be recovered, i.e., approximately 0.8 mm. from the pit. Anteriorly the areas follow closely the level of the tip of the head-process, never extending beyond it. Their posterior extent is approximately 0.4 mm. from the pit (see Fig. 6). Certain changes in the length of the areas accompany morphological changes in head-process formation.

4. A gradient in developmental potency was observed within each area. There is a center where the capacity for heart differentiation is most firmly established and where heart is produced with the highest percentage of frequency. Toward the periphery of the areas the potency diminishes, as indicated by a decrease in the frequency of heart occurrence in the grafts.

5. While the data do not indicate any striking asymmetrical differences in the shape and extent of the two areas, they do show the superiority of the left side of the blastoderm over the right in its capacity to produce both grafts and hearts, particularly where far-lateral portions of the blastoderm are concerned.

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PLATE I

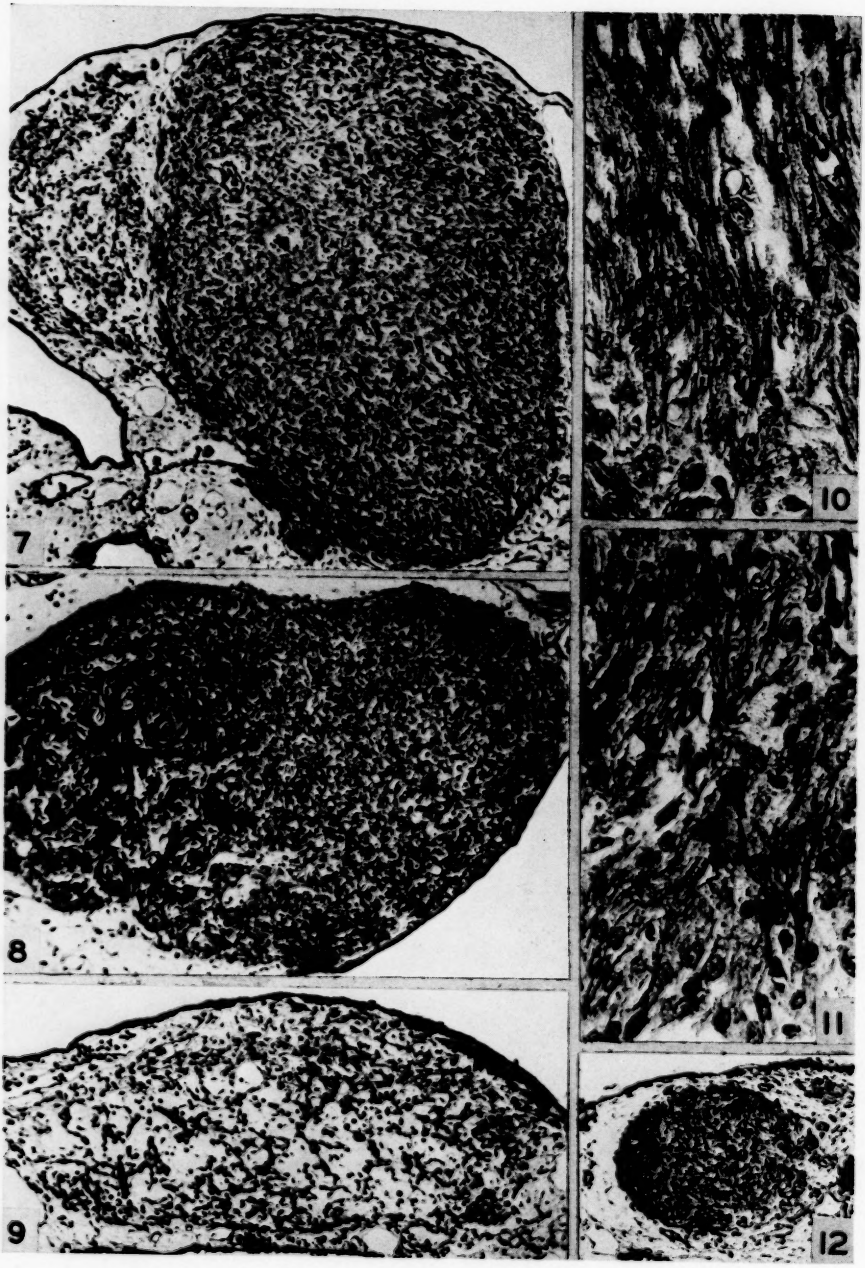

PLATE I

FIG. 7.—Section through a graft heart developed from Level D, right (see Fig. 2), showing the compact and diffuse types of cardiac-muscle differentiation in the same heart mass. × 100.

FIG. 8.—Section through a graft heart developed from Level C, right (see Fig. 2), showing a typical cardiac musculature. Note coarser fibers in the left portion of section, which further on in the series surround larger blood vessels. × 100.

FIG. 9.—Section through a graft heart developed from Level D, left (see Fig. 2), showing the diffuse type of differentiation occasionally obtained at all levels. × 100.

FIG. 10.—Longitudinal section through a normal 10-day chick heart (ventricular portion). × 450.

FIG. 11.—Section through the musculature of a typical graft heart (total age, 10 days), showing the same degree of development exhibited by the normal (control) 10-day chick heart of Fig. 10. × 450.

FIG. 12.—Section through a graft heart developed from a *L2* piece (see Fig. 5), isolated 0.62 mm. from the mid-line (primitive pit). The small size is characteristic of the hearts obtained from grafts of the outer portions of the heart areas. Note the typical structure. × 100.

AN ANALYSIS OF BALANCER DEVELOPMENT IN TRITURUS TOROSUS

(Four plates)

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IT HAS been suggested by Harrison (1925) that the balancer already determined in the young neurula depends for its development on the support of the underlying mesectodermal material of the head crest. This conclusion he based mainly on the observation that balancer ectoderm would not give rise to balancer when transplanted into the trunk region of younger tail-bud stages, where only trunk mesectoderm, but not head mesectoderm, is presumably present. The balancer fails to develop also when the balancer ectoderm is transplanted into the stomodeal region, where no mesectoderm is available. Harrison believed, therefore, that balancer formation can take place only when the balancer ectoderm is able to use mesectoderm of the head region for its developmental support. The mesectoderm of the head region is thus specific in regard to balancer formation.

In the course of earlier experiments the incidental observation was made that balancer ectoderm of *torosus* neurula gave rise to balancer when transplanted into the flank region of older *torosus* tail-bud stages. Since it was difficult to fit this fact into the interpretation Harrison had given for balancer development and since subsequent experiments had confirmed the original observation, a series of experiments was designed in order to clarify the issue. These experiments involved among others the transplantation of balancer ectoderm of neurula into different body regions, using as host a greater variety and older embryonic stages than heretofore employed by other investigators. It was soon found that the balancer development in various heterotopic positions was the result of inductive stimuli emanating from the mesodermal substratum. Moreover, it became evident that the effectiveness of the inductive stimuli differed within the various regions of the embryo and with the age of the host. Further experiments in which balancer ectoderm of different age was transplanted into various body regions of hosts of different age revealed that differences existed in the responsiveness of the ectoderm. The ability of a given graft to form balancer depends upon a definite balance between the activating and the responding systems involved.

MATERIAL AND METHODS

The experiments were performed on the embryos of *Triturus torosus;* the results reported are based on approximately seven hundred valid cases distributed throughout many different series. Not all these experimental series are actually quoted here, since some of them were in

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[2] I wish to thank Dr. V. C. Twitty for many stimulating discussions during the course of these investigations. I am also indebted to my wife for valuable help.

the nature of a survey and hence of no direct importance for the understanding of the problems under discussion.

The ectodermal pieces to be transplanted were always stained with Nile blue sulphate. The position of the graft could thus be easily distinguished and its subsequent movements clearly followed by camera lucida drawings. Figures 1–6 (Pl. I) illustrate some of the representative stages used for the transplantation. The operated animals were kept at a constant temperature of 19° C. The material was fixed in Bouin, stained with Mallory triple stain, with paracarmin as a nuclear stain.

TABLE 1

SUMMARY OF RESULTS OBTAINED BY TRANSPLANTING BALANCER ECTODERM INTO THE BALANCER REGION OF HOSTS OF VARIOUS AGES

A				B				
Donor Stage	Host Stage	Total No. of Cases	No. of Cases in Which Balancer Is Present	Donor Stage	Host Stage	Total No. of Cases	No. of Cases in Which Balancer Is Present	No. of Cases in Which Balancer Is Absent
15	21	15	15	21	39	4	4	.....
15	23	16	16	22	27	8	8	.....
15	26	9	9	22	33	5	5	.....
15	27	4	4	23	27	5	5	.....
15	30	9	9	23	23	13	12	1
15	36	2	2	23	31	5	5	.....
15	38	5	5	26	33	2	2	.....
15	43	2	2	26	39	4	4	.....
				30	39	4	4	.....
Total no. of cases	.....	.....	62	Total no. of cases	.....	.....	50	.....

RESULTS

THE EFFECT OF HOST AGE ON BALANCER DEVELOPMENT

Balancer ectoderm of a *torosus* neurula with wide-open medullary plates (Stage 15) was transplanted into the balancer region of hosts varying in age from Stage 21 to Stage 43. The results of these experiments are summarized in Table 1, *A*, from which it is seen that the grafted ectoderm gives rise to balancers in all experimental groups (Figs. 7–14). In the last group the balancer of the host (Stage 43) had already reached its maximum size at the time of the operation. A few days later the following peculiar situation arose. On one side of the head the normal host balancer was in a state of gradual resorption, while on the other side the transplanted balancer was still increasing in size. Finally, 16 days after the operation, the normal left host balancer was completely resorbed, while four well-developed balancers were present on the operated right side (Fig. 15).

The fact that balancer ectoderm older than Stage 15 can also form balancers is illustrated by another set of experiments summarized in Table 1, *B*. In these experiments balancer ectoderm from donors ranging from Stage 21 to Stage 30 was transplanted into the balancer re-

gion of hosts of varying ages. As in the previous experiments it was observed that balancers were formed in all series. These experiments are, however, not entirely conclusive, since after about Stage 23 it became increasingly difficult with the aging of the donor to remove the piece of balancer ectoderm cleanly, that is, free from adherent mesodermal cells.

In all these transplantations between animals of unequal age the time of the appearance of the balancer corresponded with that in normal donor controls, that is, was independent of the age of the host. This fact has been observed by other investigators who transplanted balancer ectoderm in unequal age combinations, namely, Harrison (1925) for *Amblystoma punctatum*, Holtfreter (1933) and Rotmann (1935) for *Triton*.

It was somewhat unexpected to note that balancers developing from neurula ectoderm on very old hosts seemed to be smaller than those formed by the same ectoderm on younger hosts; a special series of experiments was designed to test this point further. Balancer ectoderm of a neurula (Stage 15) was transplanted into the balancer region of hosts of Stages 27 and 38, using the same donor embryo for both hosts in order to assure an accurate comparison between the grafted balancers in the two cases. Two such pairs were available. The first comparison was made 10 days after the operation, at which time the grafted balancer in both hosts was well developed. The balancer developing from the graft in the older host was much smaller than the balancer which was formed by the corresponding graft in the younger host (Figs. 16 and 16′). The graft on the older host of this pair gave rise to two balancers, very small indeed when compared with the single balancer formed by the graft in the younger host of the same pair. These two animals were fixed at this stage, while the second pair, very similar to the first, was raised further and revealed that the size differences of the balancers persist throughout the balancer stage. It might be said here that usually the supernumerary balancers are normal in size (cf. Figs. 13 and 7).

TABLE 2

SUMMARY OF RESULTS OBTAINED BY TRANSPLANTING BALANCER ECTODERM INTO DIFFERENT REGIONS OF THE HOST EMBRYO

	Donor Stage	Host Stage	Graft Placed at	No. of Cases in Which Balancer Is	
				Present	Absent
A....	23	31	Balancer region	5	0
	22	27	Head	5	0
	22	31	Gills	4	0
	22	31	Pronephric region	2	2
	22	31	Somites	3	1
	22	31	Anus	4	0
	22	31	Belly	0	2
B....	15	34	Pronephric region	1	4
	15	33–34	Somites	3	4
	15	33–34	Anus	1	7
	15	33–34	Belly	0	8
Total no. of cases.	.....	.....	.....	28	28

BALANCER DEVELOPMENT IN DIFFERENT REGIONS OF THE EMBRYO

In the first group of experiments to be considered, balancer ectoderm from embryos of Stages 22–23 was transplanted into various regions of embryos at Stage 31. Seven different locations of the graft in the host may be distinguished. In Table 2, *A*, where the results are summarized, we note that balancers developed in all body regions tested, except over the lateral yolk mass of the belly.

One representative case of each set is illustrated in Figures 18–23.

These experiments were repeated with younger balancer ectoderm (from neurula, Stage 15). The results (Table 2, *B*) show that, as in the transplantation of older balancer ectoderm, balancers may develop when the graft is placed in the pronephric, the somite, and the anal region of the host. Grafts placed on the side of the yolk were again the only ones which failed to develop balancers. However, in spite of the general similarity in the outcome of the transplantation of younger and older ectoderm into the same body region, there are evidently distinct differences between these two groups of experiments. In comparing the ectoderm of Stage 22 with that of Stage 15 when placed in the anal region, for example, we observe that in the former four out of four cases developed balancers, while in the latter only one out of eight cases was positive. A somewhat corresponding situation is found in the pronephric and somite regions. In all regions the chances for balancer development seem much more favorable for the older ectoderm than for the younger. Furthermore, it was found that balancers which develop from older ectoderm in the somite region are always single balancers (e.g., Fig. 21), while the younger ectoderm in the same region gave rise to multiple balancers (Figs. 24–26).

RELATIONSHIP OF BALANCER FORMATION TO HOST AGE AND POSITION OF GRAFT

The present section deals with unexpected results encountered in the course of experiments designed to extend those related in the preceding sections. In the first of these new series, balancer ectoderm from neurulae of Stage 15 was grafted into the somite region of hosts varying from Stage 23 to Stage 37. As will be seen in Table 3, grafts placed in hosts of Stage 23 never form balancers, whereas in hosts of Stage 37 identical grafts in the same location develop balancers in all cases. In host stages intermediate in age between these two extremes balancers are found to develop from the grafts in approximately one-half of the cases. We may thus conclude that between Stage 22 and Stage 37 the situation in the somite region allowing for the realization of balancer formation by the grafted ectoderm becomes more and more favorable as older hosts are employed.

In the next group of experiments balancer ectoderm taken from donors of Stages 22 and 23 was placed in the somite region of hosts ranging between Stage 23 and Stage 35. The results of these experiments are in general the same as those in the previous series (see Table 3, *B*). The importance of host age on balancer formation is very clearly demonstrated in a set of experiments in which balancer ectoderm from a single donor (Stage 22) was transplanted into the somite region of two different hosts, one at Stage 23 and the other at Stage 35. Four such pairs were available. The graft in the older host of each pair had developed a well-formed balancer, while the graft in the younger host partner was smoothly incorporated into the lateral skin of the embryo and showed no sign of balancer formation.

If one compares the young and older ectoderm as to its capacity to form balancers in the different hosts, one finds that the older ectoderm gives rise to balancers in three out of four cases when grafted to a host of Stage 31, while the younger ectoderm grafted to hosts of Stage 33 develops balancers in only one out of four cases (Table 3). This seems

to indicate that the older ectoderm is able to express its balancer tendencies in hosts which are younger than those needed by the younger ectoderm. Table 3 also reveals that the same distinction applies when transplants are made to the anal region.

A suggestion that the formation of the balancer is also associated with the location of the graft is shown in the following: Balancer ectoderm of Stage 15 seems to form balancers much more readily in the somite than in the anal region in hosts of Stages 33–34. But older balancer ectoderm (Stage 22) forms balancers in the anal region but none in the somite region when grafted to hosts of Stage 23.

The belly region, as one may recall, was found to be unsuitable for balancer formation. Since it was observed, however, that the age of the host played such an important part in the realization of the balancer tendency, the question arose whether the failure of balancer development in this region might not have been caused by an unsuitable host age. Therefore, balancer ectoderm from Stage 15 was transplanted into the belly of hosts ranging from Stage 26 to Stage 39, with the result that no clear balancers developed in this location (Table 3, *A*). If, on the other hand, balancer ectoderm from donors of Stage 22 was grafted into the belly of older hosts, balancer development took place if the host was sufficiently old (Table 3, *B*). Very instructive in this connection were experiments in which balancer ectoderm from a single donor at Stage 23 was transplanted into the belly of two hosts of different age, one in Stage 23, the other in Stage 35.

TABLE 3

SUMMARY OF RESULTS OBTAINED BY TRANSPLANTING BALANCER ECTODERM INTO HOSTS OF VARIOUS AGES AND INTO DIFFERENT REGIONS OF THE HOST EMBRYO

A					B				
Donor Stage	Host Stage	Graft Placed at	No. of Cases in Which Balancer Is		Donor Stage	Host Stage	Graft Placed at	No. of Cases in Which Balancer Is	
			Present	Absent				Present	Absent
15	23	Somites	.....	8	22	23	Somites	.....	4
15	27	Somites	2	4	23	25	Somites	.....	8
15	33	Somites	1	3	22	31	Somites	3	1
15	34	Somites	2	1	22	35	Somites	4	.....
15	37+	Somites	10	.....					
					22	23	Anus	1	1
15	33+	Anus	1	7	22	31	Anus	4	.....
15	26	Belly	.....	11	21	23	Belly	.....	4
15	27	Belly	.....	2	22	26	Belly	.....	2
15	33	Belly	.....	4	22	31	Belly	.....	2
15	34	Belly	.....	4	22	35	Belly	2	.....
15	37+	Belly	2(?)	.....	24	26	Belly	.....	4
15	39−	Belly	1(?)	7	31	23	Belly	4	.....
					31	31	Belly	2	.....
Total no. of cases	.....	.....	19	51	Total no. of cases	.....	.....	20	26

We find that the graft in the older hosts (two pairs) had developed a balancer (Fig. 17′), while only a small ectodermal knot was formed by the graft in the younger hosts (Fig. 17).

Brief reference may be made to three additional sets of experiments in this group (Table 3, *B*). Balancer ectoderm of Stage 24 was placed in the belly of hosts at Stage 26, and balancer ectoderm of Stage 31 was placed in the belly of hosts at Stage 23 or Stage 31. Only when donors of Stage 31 were employed did the graft form balancers on young hosts, but in these cases it was somewhat questionable whether the grafts were completely free of adhering mesoderm.

Before closing this section some observations concerning the behavior of the grafts should be mentioned. In the transplantation of balancer ectoderm into hosts of various ages and into different positions of the host embryo, ectoderm pieces of about the same size and more or less square in shape were used. Since these grafts were stained with vital dye, their future position on the host embryo could be followed. It was noticed that equal grafts vary as to their shape and in the extent of the area covered by them, according to their location in the host embryo and the age of the host they happen to be grafted to. Since the observed changes in the shape of the graft are presumably the result of cell movements rather than active growth, it follows that the final spatial configuration of the grafted ectoderm is the expression of both its own inherent movement tendency and that of its new environment.

AN ANALYSIS OF BALANCER MULTIPLICATIONS

As already referred to, it was noticed that in the transplantation of balancer ectoderm into the balancer region of hosts of different age, multiple balancers were frequently observed. If we group all cases in which balancer ectoderm of Stage 15 was grafted into the balancer region of hosts ranging from Stage 21 to Stage 43, the final tabulation (Table 4, *A*) shows that with an increase in the age of the hosts an increasing percentage of cases with multiple balancers was found. Most of the multiple balancers consist of two balancers, while threefold balancers are not uncommon. Balancer clusters composed of four or five single balancers are, however, relatively rare. Usually we observe the multiple balancers as being fused together at their base and, somewhat less frequently, joined together throughout their length. In multiplications which are made up of many individual balancers we often find one or two of the balancers somewhat separated from their fused partners (Fig. 12). Occasionally we find single balancers which are heavier than normal owing to a thickening of their ectodermal layer. In such balancers irregular knots, sometimes in the form of small side branches, develop. This may be regarded as the first indication of a multiple tendency (Figs 9 and 9′). In general we may say, however, that the position of the individual balancers with regard to one another varies greatly in a multiple formation. Figures 7–14 illustrate some representative cases of these balancer multiplications.

The importance of the host age as a decisive factor in bringing about balancer multiplication is also revealed by grafting balancer ectoderm of Stage 15 into the somite region of hosts of different age. Here, again, a more frequent occurrence of multiple balancers is found in older hosts. These experiments are grouped together in Table 4, *B*, where only such cases are listed as had formed balancers.

The capacity of the ectoderm to produce multiple balancers becomes limited as the ectoderm ages. The formation of multiple balancers from ectoderm of Stage 22 or older is thus very rare indeed, irrespective of the age of the host into which these older ectoderm pieces are transplanted. This can be seen from Table 4, *C* and *D*, in which such experiments are tabulated.

Summarized briefly at this point, the experiments discussed so far show that balancer ectoderm of Stage 15 is readily inclined to form multiplications; as the ectoderm increases in age, however, this tendency is gradually lost or restricted. The age of the host is a decisive factor for balancer multiplications, since young balancer ectoderm is much more inclined to form multiple balancers in older hosts than in younger ones.

TABLE 4

SUMMARY OF RESULTS SHOWING DISTRIBUTION OF MULTIPLE BALANCERS IN VARIOUS EXPERIMENTAL SERIES

	Donor Stage	Host Stage	Number of Cases in Which Balancer Is						Total No. of Cases	Percentage of Balancers Multiple
			Questionable	Single	Twofold	Threefold	Fourfold	Fivefold		
A	15	21	1	12	.....	1	1	.....	15	15
	15	23	.....	6	5	3	.....	1	15	60
	15	26	1	3	4	1	.....	.....	9	63
	15	27	.....	2	2	.....	.....	.....	4	50
	15	30	.....	2	4	2	1	.....	9	78
	15	36	.....	.....	.....	.....	2	.....	2	100
	15	38	1	1	1	1	.....	1	5	80
	15	43	.....	.....	1	.....	1	.....	2	100
	Total no. of cases	.....	.....	.....	.....	.....	.....	.....	61	.....
B	15	27	.....	2	.....	.....	.....	.....	2	0
	15	33	.....	1	.....	.....	.....	.....	1	0
	15	34	.....	1	1	.....	.....	.....	2	50
	15	37	.....	2	5	1	2	.....	10	80
	Total no. of cases	.....	.....	.....	.....	.....	.....	.....	15	.....
C	22	27	.....	6	1	1	.....	.....	8	25
	23	27	.....	5	.....	.....	.....	.....	5	0
	Total no. of cases	.....	.....	.....	.....	.....	.....	.....	13	.....
D	22	31	.....	3	.....	.....	.....	.....	3	0
	22	35	.....	4	.....	.....	.....	.....	4	0
	Total no. of cases	.....	.....	.....	.....	.....	.....	.....	7	.....

A possible explanation of the role played by the old host in bringing about balancer multiplication is derived from a further observation not as yet mentioned. It was noticed that the grafted ectoderm in the balancer and somite region of older hosts is restricted in its movement much more than in younger hosts. Since it was further found that in

such older hosts balancer multiplication occurred much more frequently, a correlation between these two facts seemed to be indicated. One might well imagine that the conflict obviously existing between the opposing movements of the graft and the host ectoderm disturbs the single centered field of the balancer anlage. This may lead to the segmentation of the field, thus causing the formation of multiple balancers.

Although the spatial configuration of the graft in older hosts seems definitely associated with the formation of multiple balancers, there are obviously still other decisive factors to be taken into account. We find, for example, multiple balancers formed by balancer ectoderm of Stage 15 in the balancer region of hosts of Stage 21, in spite of the fact that in this combination the configuration of the graft seems to be more normal. It is obvious that in hosts where the ectoderm graft is restricted in its movements the orientation of the field with regard to its environment is also affected. Thus it was thought that the orientation of the graft might be a decisive factor in bringing about multiplication. Experiments already reported seem to confirm these expectations. We find that ectoderm grafts in older hosts, which are restricted in their movement and are hence wrongly oriented, practically always form multiple balancers. Ectoderm grafts in younger hosts, which are able to express their movement more freely, form multiple balancers only occasionally, presumably because the ectoderm pieces, grafted in random orientation, may have often been placed in normal orientation.

These considerations immediately suggest an investigation of the role of the orientation of the graft in the formation of multiple balancers. Balancer ectoderm of Stage 15 was therefore transplanted into the balancer region of hosts of varying age: (1) the graft was placed in normal orientation, (2) it was rotated 180°, and (3) ectoderm from the left side of the donor was grafted to the right side of the host. The results of these experiments are summarized in Table 5. In the group of experiments in which the balancer ectoderm was normally oriented we find only single balancers formed when placed in hosts of Stage 15. An increasing number of cases with multiple balancers was found as the embryos used as hosts became older. When the balancer ectoderm is grafted in wrong orientation, it is found that the percentage of cases with multiple balancers in all series has greatly increased in comparison with the same series where the grafts are normally oriented. This fact is strikingly demonstrated, if one compares the experiments in which the balancer ectoderm was transplanted into hosts of Stage 21 in normal and abnormal orientation. Only 30 per cent of the cases formed multiple balancers when the graft was normally oriented, whereas 85 per cent of the cases in the "left and right inversion series" and 100 per cent of the cases in the "180° rotation series" produced multiple balancers.

The results of these experiments provide strong evidence that the orientation of the graft is a decisive factor in producing multiple balancers. In experiments where graft and host are of equal age, the graft forms single balancers when oriented normally but multiple balancers when its orientation is reversed. If the grafted ectoderm is younger than the host its movements are restricted, and because of this its relation to the older host necessarily becomes abnormal. This condition results in multiple balancers, although the original orientation of the graft was normal. The greater the age

difference between donor and host, the more often multiple balancers occur, even though the graft was normally oriented (see Table 5). This is due to the fact that the differences in the movements between donor and host ectoderm become more and more pronounced as the difference in age between donor and host becomes greater.

From previous experiments it was concluded that the ability of the ectoderm to form multiple balancers decreases as the ectoderm ages. Since the results of these experiments were obtained from grafts which were oriented at random, it is of interest to reinvestigate these findings, using grafts of known orientation. Accordingly, a number of experiments was designed, the results of which are summarized in Table 5. In all cases listed, the grafts were placed in the balancer region. In general, one has the impression that multiplications are less readily formed by older ectoderm, because even abnormally oriented ectoderm of Stages 23 and 26 forms single balancers in hosts of Stage 31. If, however, the age difference between donor and host is large enough, multiplication may be observed from ectoderm of Stage 30. This indicates that segmentation can take place in an ectoderm as old as Stage 30 and that the segments are still able to produce a harmonious organ.

TABLE 5

SUMMARY OF RESULTS SHOWING THE INFLUENCE OF ORIENTATION OF THE GRAFT ON THE FORMATION OF MULTIPLE BALANCERS

Donor Stage	Host Stage	Orientation of Graft	No. of Cases Where Balancer Is			Total No. of Cases	Percentage of Balancers Multiple
			Single	Twofold	Threefold		
15	15	Normal	8	.....	.....	8	0
15	21	Normal	9	2	2	13	30
15	24	Normal	6	1	1	8	25
15	30	Normal	5	5	2	12	58
15	15	l/r inv.	1	.....	1	2	50
15	21	l/r inv.	2	6	5	13	85
15	15	180° inv.	2	.....	.....	2	0
15	21	180° inv.	.....	4	1	5	100
15	23	180° inv.	.....	2	.....	2	100
21	39	Normal	3	1	.....	4	25
26	39	Normal	3	1	.....	4	25
30	39	Normal	1	3	.....	4	75
22	33	l/r inv.	2	3	.....	5	60
23	31	l/r inv.	5	.....	.....	5	0
26	33	l/r inv.	2	.....	.....	2	0
23	23	180° inv.	12	.....	.....	12	0
Total no. of cases	.....	.....	.....	.....	.....	101	.....

THE RELATIONSHIP BETWEEN INDUCTION AND REACTION IN THE DEVELOPMENT OF THE BALANCER

Mangold (1933) has shown that balancer ectoderm from *Triton taeniatus* neurulae (Stage 15) isolated *in vitro* in

Holtfreter's solution is unable to form balancers. Following isolation these ectoderm pieces usually round up or become attached to the bottom of the culture dish, forming loosely folded masses of epithelial cells atypical in character. When this experiment was repeated with *Triturus torosus* (8 cases), the same result was obtained. Figure 28 illustrates a typical case, sectioned 12 days after the isolation. Two alternative explanations of the failure of the balancers to develop in these experiments are possible: (1) the balancer ectoderm of Stage 15 is not yet determined and (2) something in the organic environment of the embryo necessary for the realization of the balancer is missing *in vitro*. The first alternative is disproved by the fact that balancer ectoderm of Stage 15 is, under certain conditions, able to develop balancers in the flank of host embryos and must hence already have been determined.

These experiments also provide some information regarding the time at which the balancer determiners (inductors) exert their influence upon the ectoderm in that they show that the inductors must already have acted prior to Stage 15. The question still remains, however, whether the balancer region of Stage 15 is still capable of inducing balancers and, if so, how long this inductive power persists. To this end ectoderm of the belly region of neurulae (Stage 15) was grafted into the balancer region of hosts ranging in age from Stage 15 to Stage 35. It was found (Table 6) that only hosts of Stages 15–21 are able to induce balancers in a few cases, while in older hosts the inductive capacity of this region is lost or so much weakened that it can no longer cause the formation of balancers. The induction of balancers from trunk ectoderm in the balancer region was also observed by Mangold (1931), who grafted *taeniatus* trunk ectoderm of young neurulae into the balancer region of *A. mexicanum* neurulae.

Experiments in which belly ectoderm from donors of Stage 24 was transplanted into the balancer region of hosts of Stage 21 (18 cases) proved to be all negative. In this series there were some cases in which the grafted piece did not completely cover the balancer region of the host. Host balancers would develop in such cases; but their development was more or less handicapped, depending upon the extent to which the foreign ecto-

TABLE 6

SUMMARY OF RESULTS OBTAINED BY TRANSPLANTING BELLY ECTODERM OF STAGE 15 INTO THE BALANCER REGION OF HOSTS OF VARIOUS AGES

Host stage	15	21	22	23	27	34	35
No. of cases where balancer is present	1	1	...	...	...	...	...
No. of cases where balancer is absent	6	1	4	5	3	7	8
Total no. of cases	7	2	4	5	3	7	8

derm had covered the balancer area. At the side where the graft bordered the normal balancer area some of the grafted material was always incorporated into the developing balancer. Obviously, the balancer had some difficulties in compelling the grafted ectoderm to collaborate with its rapid growth, and as a consequence it was bent over to the side of the graft. The more foreign ectoderm that was incorporated into the host balancer, the smaller and the more bent the resulting balancer happened to be (Fig. 27). This situation was not only observed in grafts of this age but also occurred when grafts of belly ectoderm of Stage 15 were used, which did not completely cover and thus did not suppress the normal host balancer.

The failure of balancer neurulae ecto-

derm to develop balancers *in vitro* was explained by the assumption that some specific cellular material present in the embryo and necessary for the development of the balancer is lacking *in vitro*. However, the question as to the exact nature of this material remains to be answered. The first thought which comes to our mind in considering the situation is that the lack of mesectoderm or mesentoderm in these experiments is the responsible factor. For it has been proved that balancer ectoderm of the neurula, when isolated together with head neural crest (Fig. 29) (Mangold, 1933) or with rostral-lateral mesoderm (Mangold, 1933) or when transplanted into the head, somite, or anus region of hosts at Stage 39, will frequently form balancers. Therefore, it seems as though the absence of mesectoderm or mesentoderm in isolated balancer ectoderm of Stage 15 is responsible for the failure of balancer development, since balancers develop when either of these two materials is present. Yet we find to our surprise that in the transplantation of the same balancer ectoderm into the somite region of young hosts no balancers developed, in spite of the fact that an ample supply of mesoderm or mesectoderm must have been present in this region. The answer to this is revealed in the histological examination of the cases in question.

To be considered first are experiments in which balancer ectoderm of Stage 15 was grafted into the somite region of hosts ranging from Stage 23 to Stage 37. The results of these experiments are summarized in Table 7, where it is seen that, in addition to balancers, the graft had given rise to a variety of neural tissues. Viewing these experiments as a whole, we note that large neural complexes and neural tubes (Figs. 30 and 31) are relatively seldom formed. More often neural vesicles appear, which develop as neural invaginations from the ectoderm, being finally pinched off as development proceeds, thus forming larger or smaller neural vesicles, usually located close to the grafted ectoderm (Figs. 30 and 32). Small complexes of neural cells resembling neural ganglia, as well as neural thickenings of the ectoderm and ectodermal sense organs, are frequent (Figs. 32–34). The amount of mesenchyme under the grafted area is always increased, and the grafted ectoderm is usually abnormally thickened. Thus, in general, we observe in these experiments various neural formations. If we now compare the distribution of these neural formations within the different experimental series, we find them arranged in a certain order. Large neural complexes and neural tubes are formed only in younger hosts of Stages 23–27. In hosts of Stage 33 neural vesicles are formed, while in hosts older than this we observe only loosely arranged neural cells (ganglia) or ectodermal thickenings of neural character. With an increase in the age of the host, we thus observe a decline in the quality and quantity of neural formation. Directing attention again to Table 7, we notice one further point of particular interest to our problem, namely, that with a decline in the quality of the neural induction the number of cases in which balancers are formed increases. Or, to state this fact somewhat differently, in the presence of large neural formations balancers are lacking, whereas small neural formations are accompanied by balancer development.

When balancer ectoderm of Stage 15 or Stages 21–24 was transplanted into the belly region of hosts of different age, it was found in sectioning these cases that the ectoderm was usually somewhat

thickened in the region of the graft and the mesoderm increased (Fig. 35), but no neural structures were formed (see Fig. 7).

In another series of experiments belly ectoderm of neurulae (Stage 15) was transplanted into the somite region of hosts of Stage 35 (8 cases) and Stage 39 (6 cases). In none of these cases did the host embryo, while the same ectoderm is unable to form these structures when placed in a noninductive host region (belly) or when cultured *in vitro*. From this we must conclude that balancer formation from the grafted balancer ectoderm is brought about as the result of inductive stimuli by the substratum and is not the expression of self-differentiat-

TABLE 7

DIFFERENTIATION ACHIEVEMENTS OF THE BALANCER ECTODERM IN VARIOUS EXPERIMENTAL SERIES

Donor Stage	Host Stage	Graft Placed at	Total No. of Cases	No. of Cases in Which Graft Has Formed								
				Balancer	Neural Complexes	Neural Tubes	Neural Vesicles	Neural Cells	Neural Thickening of Ectoderm	Ectodermal Sense Organs	Ectodermal Thickening	Mesenchyme
15	23–	Somites	7	.....	1	1	6	6	4	7	5	7
15	27–	Somites	6	1	2	1	4	5	5	6	3	6
15	33	Somites	4	1	.....	.....	1	3	3	4	3	4
15	34	Somites	3	2	.....	.....	.....	3	3	3	2	3
15	37	Somites	10	10	1?	.....	.....	7	3	10	10	10
15	27	Yolk	2	.....	.....	.....	.....	.....	.....	.....	2	.....
15	33	Yolk	4	.....	.....	.....	.....	1?	1	.....	4	2
15	34	Yolk	4	.....	.....	.....	.....	.....	.....	.....	4	1
15	37	Yolk	2	.....	.....	.....	.....	.....	.....	.....	2	2?
23	23	Somites	4	.....	.....	.....	.....	4	.....	4	.....	4
23	25	Somites	8	.....	.....	.....	2	4	.....	2	.....	5
22	31	Somites	4	3	1	.....	.....	4	4	4	4	4
23	35	Somites	4	4	3	.....	2	3	4	3	.....	4
21	26	Yolk	4	.....	.....	.....	.....	.....	.....	.....	4	.....
24	26	Yolk	4	.....	.....	.....	.....	1?	4	1?	4	3
23	23	Yolk	2	.....	.....	.....	.....	.....	.....	.....	2	2
23	35	Yolk	2	2	.....	.....	.....	.....	.....	.....	.....	2?

graft form balancer or, as the histological examination revealed, any neural structures.

DISCUSSION

THE DEPENDENCE OF BALANCER DEVELOPMENT ON INDUCTION

We have seen that balancer ectoderm of *torosus* neurulae differentiates into various neural structures and balancers only when transplanted into inductive regions, such as head, trunk, or tail of the ing tendencies of the graft. However, the situation is not so simple as this, for we find none of these structures developing when belly ectoderm, instead of head ectoderm, from young neurulae (Stage 15) is placed in the inductive somite region of older tail-bud stages. Therefore it is evident that already, prior to the neural-plate stage, a definite inclination for balancer development is established in the head ectoderm. This inclination, however, can be expressed only in the

presence of an inductive impulse. This leads us directly to the question whether or not we have to regard the various induced neural structures, too, as such reinforced self-differentiations. This seems probable, indeed, for the anlagen of such structures as sense organs and neural ganglia may have been included in the transplanted piece of head ectoderm.

As far as the larger neural inductions are concerned, their origin is somewhat more difficult to trace. It was noticed that some of the induced large neural complexes closely resembled nasal tissue. This similarity was more pronounced in structures developing from older grafts (Stage 22 and older), where nasal pits could sometimes be clearly distinguished. It thus seems possible that the large neural inductions and neural tubes represent more or less distorted nasal placodes, the rudiment of which had been included in the ectodermal pieces used for the graft. Also the various neural thickenings of the ectoderm may be nothing more than early abortions of olfactory organs. When we compare the neural structures formed by balancer ectoderm of Stage 15 with those formed by balancer ectoderm of Stage 22, we observe that the frequency with which certain inductions occur is different in the two ectoderms (Table 7). It must be realized here that the topographical pattern of the various anlagen changes very much in these early stages of development. It is therefore possible that supposedly equal pieces of balancer ectoderm taken from different stages may include different organ rudiments.

When balancer ectoderm was transplanted into the belly—that is, into the lateral yolk-mass of hosts—it was assumed that the graft was placed in a region lacking any inductive power. This is not quite correct, because the grafted pieces in this position are in contact with the lateral mesodermal plates in younger hosts. As development proceeds, however, the lateral tips of the somites gradually extend downward over the lateral surface of the yolk, forming the muscles of the belly wall, while the lateral mesodermal plates give rise to the peritoneal lining of the body cavity. Therefore, if we graft the ectoderm laterally into the belly of older stages, it is placed over the lateral surface of the somite tips. The different location of the graft in respect to its substratum in younger and older hosts may very well affect the outcome of the experiments and must therefore be taken into account. As far as the balancer inductions in the belly are concerned, we find that balancer ectoderm of Stage 15 is unable to form balancers in the belly region in hosts of all ages tested (Table 7). This indicates that neither the lateral plates nor the lateral tips of the somites are capable of releasing inducing stimuli strong enough to compel this ectoderm to form balancer. However, balancer ectoderm of Stage 23, when grafted into the belly, will form balancers in hosts of Stage 35 but not in younger hosts. This shows that the inductive strength of the lateral somite tips is stronger than that of the mesodermal side plates and that the inducing stimuli of the lateral somites are strong enough to assure balancer development from older but not from younger ectoderm. From this it becomes evident that the relationship between the strength of the induction, on one hand, and the readiness to respond of the ectoderm, on the other, is the decisive factor in determining whether or not balancer formation will take place.

THE SPECIFICITY OF THE INDUCTION

The next question which remains to be answered is why balancers are not formed from balancer ectoderm when

placed in the somite region of younger hosts. The reason for this is especially difficult to understand in view of the previous evidence that balancer ectoderm needs an inductive impulse for the expression of its balancer tendencies. This impulse should be readily supplied by the young somites, because their inductive properties are supposedly much better than those of the older somites. Now we find that the frequency with which larger neural inductions occur seems to decrease with an increase in the age of the host, while at the same time the frequency of balancer inductions increases. A correlation between the strength of the induction and the type of reaction thus becomes evident. It seems as if inductive stimuli of definite strength call forth definite organs. Strong inductive stimuli provided by the young somites may cause the formation of neural structures and in some way suppress balancer formation, while weaker inductive stimuli, emanating from the older somites, promote balancer formation and restrict neural inductions. However, if the inclination of the ectoderm for balancer formation has become more pronounced, by leaving the ectoderm longer in its own environment, that is, in using balancer ectoderm from older stages (Stage 22) for the transplantation, we observe that these ectoderm pieces will form balancers in hosts much younger than those needed for the same task by the younger ectoderm (Table 7). The presence or absence of balancers in our experiments thus seems to depend upon the competition between the strength of the inductive demand and the strength of the balancer inclination in the reactive system.

Since we have seen that the balancer ectoderm is able to express its balancer tendencies only in locations possessing inductive properties and since the balancer region must be regarded as such an inductive region, the development of balancers here is obviously also brought about by induction. Now we find in our experiments that hosts of all ages induce balancers in the balancer region, in contrast to the balancer induction in the somite region. This is best explained by the assumption that the balancer region for a long time maintains an inductive level high enough to promote balancer formation.

There is, however, another way of looking at the whole problem under discussion. Such structures as neural fragments, neural placodes, ectodermal sense organs, and ectodermal thickenings of neural character might well have been brought about as the result of specific inductive demands emanating from the mesodermal somites of the trunk region. Now it has been known that the competence of the presumptive gastrula ectoderm to form neural tissues by induction becomes gradually weakened as development proceeds and seems to be lost when the ectoderm reaches the neural-plate stage (Mangold, 1929). Yet Holtfreter (1938) described experiments in which he showed that ectoderm even older than that used by us may, under certain conditions, still be competent to the task of being induced. He isolated *Triton taeniatus* gastrula ectoderm in Holtfreter's solution and transplanted those pieces into the inductive trunk region of tail-bud hosts, after these pieces had been cultured *in vitro* for various lengths of time. Of special interest for our problem are those ectodermal pieces which were cultured as long as 19–21 hours, because by that time the ectoderm, if left in the embryo, would have been the equivalent of neurula ectoderm of about Stage 15. As far as neural inductions were concerned, the ectoderm could give rise to regional specific structures such as neural vesicles,

neural cells, and neuroids. These regional specific inductions observed by Holtfreter often resembled closely some of our neural inductions obtained by grafting balancer ectoderm of Stage 15 into the somite region of hosts of older tail-bud stages. From this it seems possible, indeed, to regard some of these neural structures as regional specific inductions. If this interpretation is correct, we must assume that the young hosts elicit regional specific inductive stimuli which are strong enough to overcome completely the inclination of the head ectoderm to respond to inductive stimuli with balancer formation, while the obviously weaker inductive demands of the older hosts cannot suppress the balancer tendencies of the transplanted ectoderm. This, then, explains why in our experiments balancers are formed by balancer ectoderm of neurulae in the somite region of older but not younger hosts. We might expect that older balancer ectoderm should have been more firmly determined and thus more readily capable of forming balancers; in other words, older ectoderm should oppose even strong, specifically directed, inducing stimuli, thus being able to enforce its own balancer tendencies better than the younger ectoderm. Experimental evidence for this is given in comparing the results obtained from the transplantation of balancer ectoderm of Stages 15 and 22 into the somite region of hosts of Stages 33 and 31, respectively (Table 7). One finds that the younger ectoderm gives rise to balancers in 25 per cent and the older ectoderm in 75 per cent of the cases. From this we may infer that certain tendencies already impressed upon the ectoderm become more and more hardened and hence less and less able to be changed as the animal increases in age. The inductive impulse, although specific in nature, may be answered by the reactive system in the only way possible for it, namely, by the realization of its predisposed tendencies. Specific inductive stimuli may produce regional unspecific organs, depending upon the competition between the strength of the specific inductive demand and the strength—that is, the depth—of the previously imprinted pattern in the reactive system. A specific induction may thus express itself as an unspecific one, indicating the difficulties sometimes encountered in determining the quality of an induction from a reaction.

The fact that balancer ectoderm is always impelled to form balancers in the balancer region of hosts of every age tested may easily be explained in the light of the foregoing considerations. The inductive impulse of the balancer region is obviously balancer specific in nature. No competition therefore exists in this region between a specific induction and a specific inclination of the reactive system. On the contrary, the balancer ectoderm is greatly supported in its task of expressing its balancer tendencies by inductive demands balancer-specific in nature.

SUMMARY

Homoplastic and homoplastic-anaplastic transplantations in various combinations were performed on the embryos of *Triturus torosus* in order to analyze the development of the balancer. The main results are as follows:

1. Facial ectoderm of the young neurula, including the presumptive balancer region, will form balancer when grafted to the balancer region of hosts ranging in age from Stage 15 to Stage 43. The time of appearance of the balancer, as well as the time of its resorption, is determined by the graft in all these combinations.

The size of the balancer and its rate of development is also determined by the ectoderm.

2. The formation of balancers from balancer ectoderm of Stages 21–22 takes place in the head, the gill, the pronephric, the somite, and the anal regions when the ectoderm is grafted to hosts of Stage 31. Balancer ectoderm of Stage 15 will also form balancers in the pronephric, the somite, and the anal regions of hosts of Stage 34. However, the formation of balancers in these regions by the younger ectoderm is less frequent than that of the older ectoderm. Neither the ectoderm of Stage 15 nor that of Stages 21–22 will form balancers in the belly of hosts of Stages 31–34.

3. The formation of balancers in the somite region from balancer ectoderm of the young neurula depends upon the age of the host. In the somite region of hosts of Stage 23 no balancer development occurs, while the same graft forms balancers in 100 per cent of the cases in the somite region of hosts of Stage 37. The same situation is found when older ectoderm is used as graft. The older ectoderm, however, is able to form balancers in hosts which are younger than those needed for balancer formation by the younger graft. The importance of a definite age relationship between donor and host as a decisive factor for balancer development is characteristic not alone of the somite region but of other regions as well.

4. The formation of balancers is also associated with the location of the graft on the embryo. Certain regions are more favorable than others for balancer development.

5. An analysis of balancer multiplication has revealed that the orientation of the graft is a decisive factor in producing multiple balancers.

6. The balancer ectoderm is already determined in the young neurula but needs for its realization an inductive impulse unspecific in nature. The presence or absence of balancers in our experiment depends upon the competition between inductive and reactive system. The relation between induction and reaction in the determination of the balancer is discussed in some detail.

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PLATE I

FIGS. 1–6.—Representative stages employed in the experiments: Fig. 1, Stage 15; Fig. 2, Stage 22; Fig. 3, Stage 26; Fig. 4, Stage 30; Fig. 5, Stage 33; Fig. 6, Stage 37.

FIG. 7.—Normal *torosus* embryo at Stage 39.

FIGS. 8–14.—Various cases with balancer multiplication obtained by transplanting balancer ectoderm of Stage 15 into the balancer region of older hosts. Fig. 8, single balancer; Fig. 9, balancer abnormally heavy; Fig. 10, double balancer; Figs. 11 and 12, balancer threefold; Fig. 13, balancer fourfold; Fig. 14, balancer fivefold; Fig. 9′, sections through balancer of Fig. 9; Fig. 14′, sections through fivefold balancer of Fig. 14.

PLATE II

FIG. 15.—Transplantation of balancer ectoderm of Stage 15 into the balancer region of a host of Stage 43. (*Note:* Normal balancer on left side already resorbed, while four balancers are formed on right side by graft.) Photograph, 16 days after operation.

FIGS. 16 AND 16′.—Simultaneous transplantation of balancer ectoderm of Stage 15 into two hosts. Fig. 16, host, Stage 27; Fig. 16′, host, Stage 38. (*Note:* Formation of larger balancer on younger host.)

FIGS. 17–17′.—Simultaneous transplantation of balancer ectoderm at Stage 22 into the belly of two hosts. Fig. 17, host, Stage 23; Fig. 17′, host, Stage 35. (*Note:* Balancer only formed in older host.)

FIGS. 18–23.—Transplantation of balancer ectoderm of Stage 22 into different regions of hosts of Stage 34. Fig. 18, into head; Fig. 19, into gill region; Fig. 20, into pronephric region; Fig. 21, into somite region; Fig. 22, into anal region; Fig. 23, into belly. (*Note:* Balancer formed in all regions except belly.)

PLATE III

FIGS. 24–26.—Transplantation of balancer ectoderm of Stage 15 into the somite region of hosts of Stage 37. Fig. 24, fourfold balancer formation; Fig. 24′, section through case of Fig. 24 (*Note:* Secretion cones on fused balancer); Fig. 25, fourfold balancer formations (case somewhat older than Fig. 24); Fig. 26, double balancer formation; Fig. 26′, section through case Fig. 26.

FIG. 27.—*Right:* chimeric balancer obtained by transplanting belly ectoderm of Stage 24 into balancer region of a host of Stage 21. (*Note:* Balancer bent over; stippled line indicates border of grafted ectoderm.) *Left:* normal control side.

FIG. 28.—Sections through isolated balancer ectoderm of Stage 15 in Holtfreter solution. (*Note:* No balancer formation.)

FIG. 29.—Isolation of balancer ectoderm plus head crest in Holtfreter solution. (*Note:* Formation of typical balancer.)

PLATE IV

FIGS. 30–34.—Sections illustrating some typical inductions obtained by grafting balancer ectoderm into the somite region of hosts of different age (see text): *ind.*, large neural induction; *n.t.*, neural tube induction; *n.v.*, induced neural vesicle; *n.c.*, induced neural cell nest (ganglia?); *e.th.*, induced thickening of ectoderm of neural character; *s.b.*, induced sense bud; *b.*, induced balancer.

FIG. 35.—Transplantation of balancer ectoderm of Stage 15 into belly region of young host. (*Note:* Abnormal thickening of the ectoderm.)

PLATE I

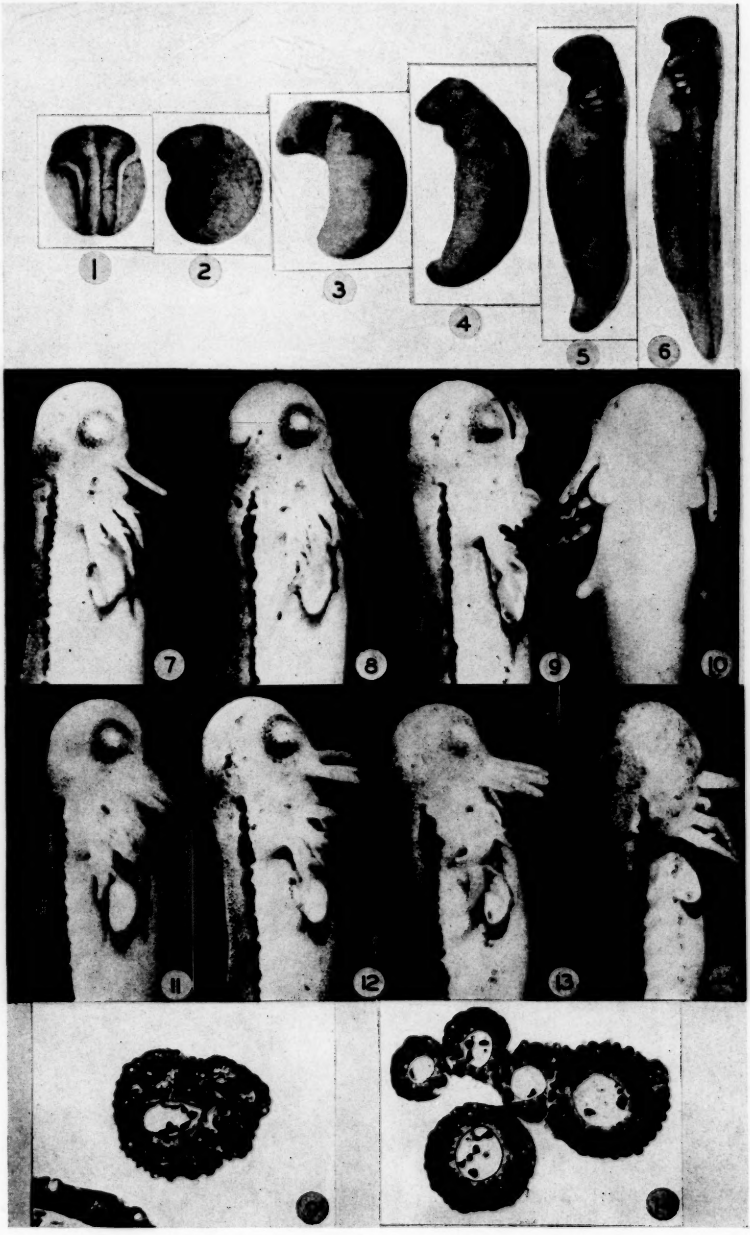


PLATE II

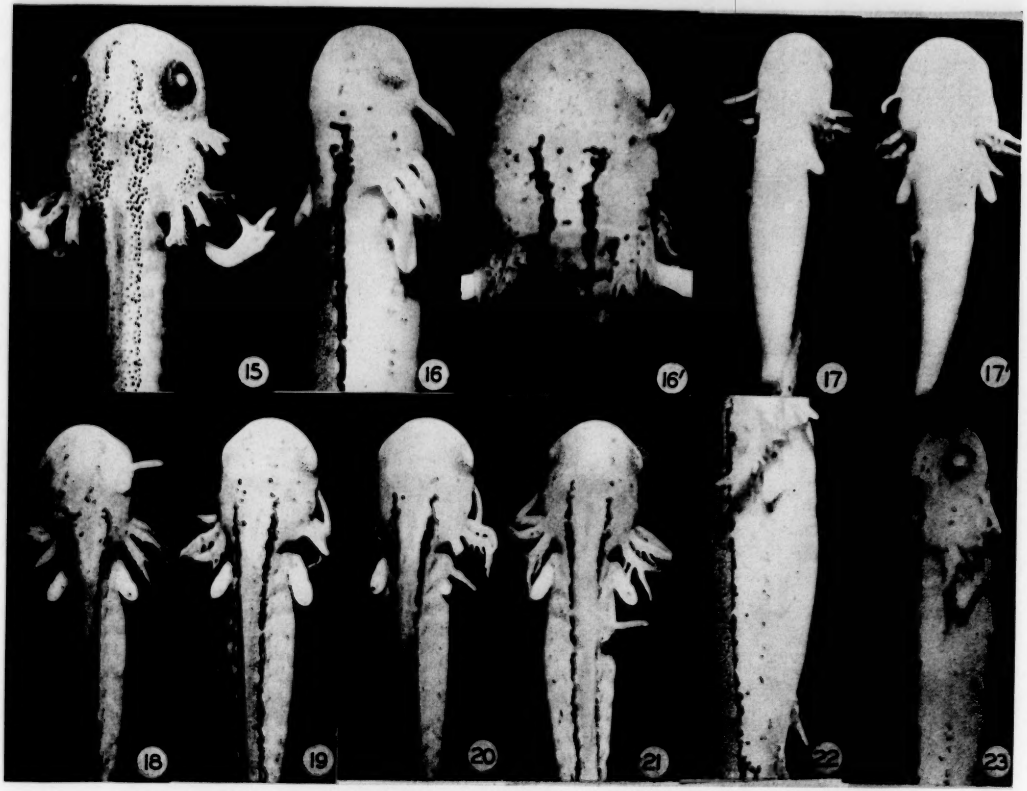


PLATE III

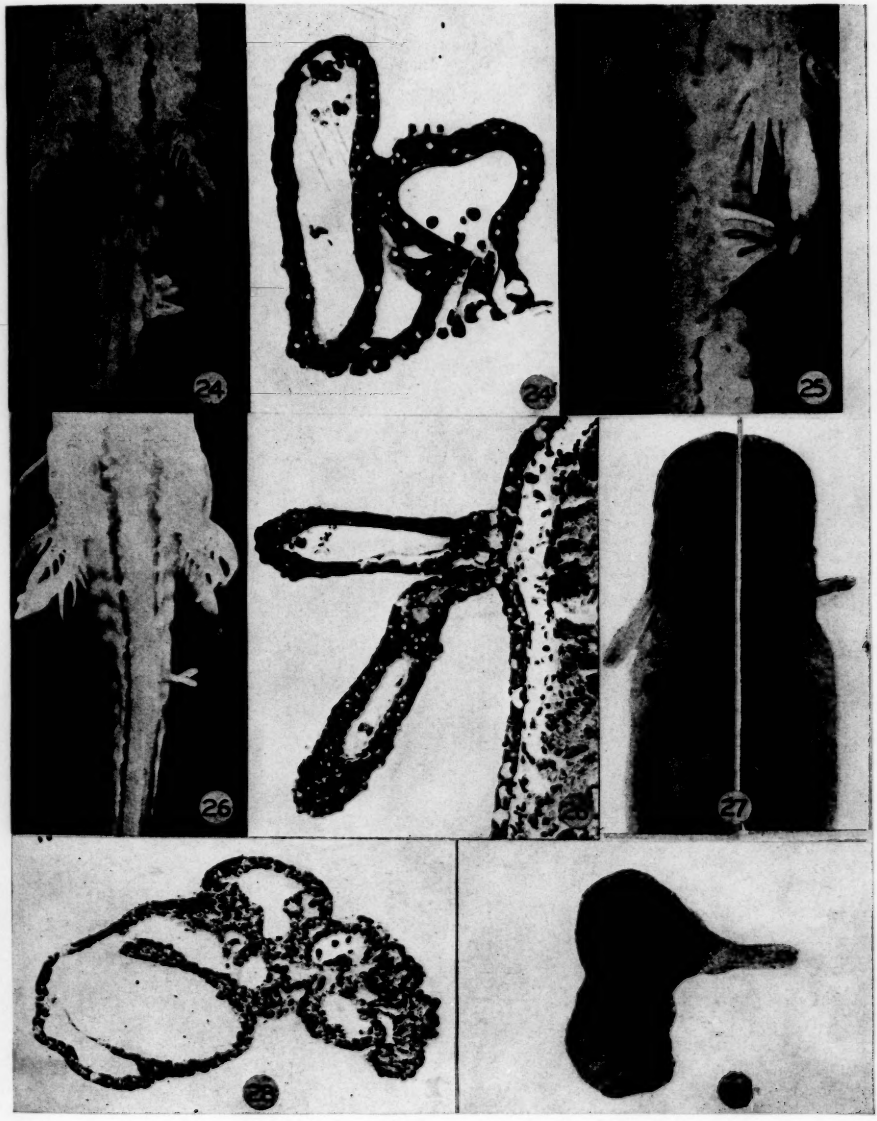
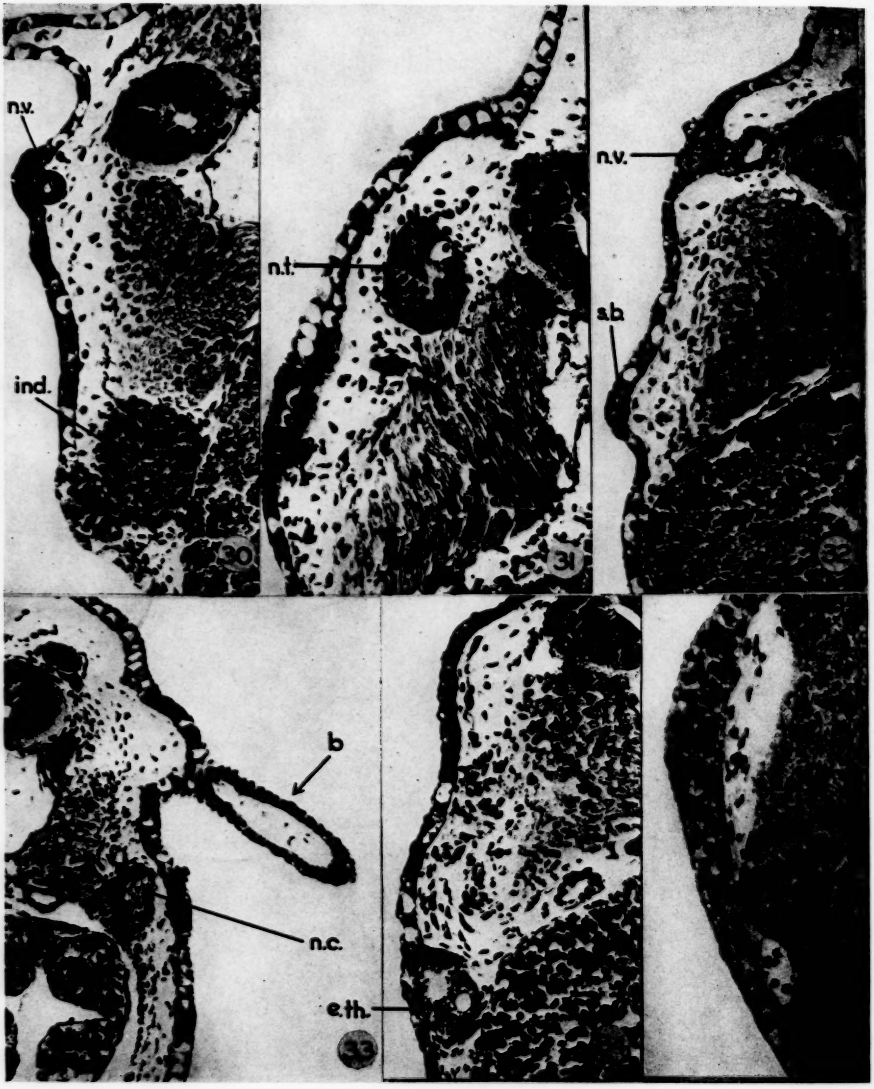


PLATE IV



DIFFERENTIAL DYE REDUCTION AND REOXIDATION IN TRITURUS DEVELOPMENT

(Eighteen figures)

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THE question whether definite regional or graded differences in rate of respiration or other metabolic activities are characteristic of early embryonic amphibian development has received considerable attention during recent years. Various investigators have attempted to throw light on the problem of the nature of neural induction by the dorsal inductor region, the so-called "organizer," by studies on the metabolism of this region as compared with other parts of the embryo. More than twenty years ago differences in susceptibility to various agents, indicating a decrease from the anterior (animal) pole and a higher susceptibility appearing on the dorsal side and attaining a maximum in the dorsal lip of the blastopore were observed in the frog embryo (Bellamy, 1919). More recently determinations of oxygen consumption, CO_2 production, respiratory quotients, glycolysis, and some data on distribution of glycogen and sulphydryl proteins have appeared. Since these have recently been discussed at some length with a bibliography (Child, 1941, pp. 151–58), extended consideration is not necessary here. Although the different lines of evidence from these investigations are not in complete agreement, they do indicate the presence of a definite spatial developmental pattern in which metabolic activity of some kind is evidently a factor. The significance of determinations of oxygen uptake and CO_2 production of dorsal and ventral regions of single embryos, showing higher respiration dorsally, has been regarded as uncertain because of the presence, as invagination proceeds, of two cell layers on the dorsal side and because some determinations on isolated pieces from the dorsal lip and the ventral region do not show such differences. On the other hand, it has been pointed out that determinations of oxygen uptake and CO_2 production on isolated pieces are of uncertain significance because of regional differences in yolk content of the cells concerned. In pieces which show the same oxygen uptake or CO_2 production per unit of weight and time (QO_2), proportions of yolk and of respiring protoplasm and consequently the real rates of respiration may be very different. Moreover, the significance of data on respiration in isolated pieces is perhaps open to question on the ground that oxygen uptake, CO_2 production, and glycolysis may be more or less altered by the cell injury or other effects associated with, or following, isolation, so that they are no longer indicative of these activities in different regions of the intact embryo. In view of evidence from various other organisms, it is not impossible that a region of higher respiratory rate may be more susceptible to stimulation or damage involved in isolation than one of lower rate. If isolation does alter respiratory rates, its effect may increase with decrease in size of isolated piece. Considering these possibilities, it appears

that the data obtained from single, very small isolated pieces with the Cartesian diver "ultrarespirometer" (Boell, Needham *et al.*) may be less rather than more significant than determinations from a number of larger pieces from the same regions of different individuals (Barth, 1942). With the larger pieces effects of isolation are perhaps less extreme, and individual differences incidental to operative procedure or of other character may be more or less balanced when several pieces are used.

The present paper is a study of differential reduction and reoxidation of methylene blue, with some data on Janus green, in living, intact embryos and larvae of *Triturus torosus* and *T. rivularis*. The results obtained with dyes do not prove that any of the determinations of respiration on isolated pieces are incorrect, though they perhaps suggest that such determinations do not tell the whole story.

MATERIAL AND METHODS

Eggs from different females of *T. torosus* and *T. rivularis* differ widely in depth of pigmentation. The more deeply pigmented eggs are useless for work with dyes, but lightly pigmented egg masses occur not infrequently, and completely pigmentless, albino egg masses are occasionally found. This study has been made possible by the kindness of Dr. V. C. Twitty in permitting selection of lightly pigmented material in various stages from his collections and in providing one albino lot of *T. rivularis* in an early stage. I gratefully acknowledge my deep indebtedness.

In 1938 an attempt was made to study dye reduction in *Triturus* embryos; but, if the embryos were left within the vitelline membrane, dye penetration was even slower than after removal of the membrane. Dye reduction was brought about by allowing the embryos to decrease oxygen gradually by their own oxygen uptake in a small amount of dye solution sealed from air. Gradual oxygen decrease in this way was regarded as preferable to exposure at once to completely anaerobic conditions for showing any regional differences in rate of dye reduction which might be present. After removal of the jelly, embryos within the vitelline membrane can be moved about in the sealed preparation by change of position with respect to gravity in order to maintain uniformity of oxygen content in the solution. Naked embryos of earlier stages are extremely fragile, easily injured, and tend to flatten on the substrate so that they can be moved about and turned over to insure uniform staining or uniform oxygen content in solution during reduction only with great difficulty or not at all. Consequently, in these early stages reduction could be observed only over part of any one embryo, and there was no certainty that oxygen decrease in the solution was uniform. In spite of these difficulties a few data were obtained which indicated definite regional differences in dye reduction, a decrease in rate of reduction from the apical or anterior pole in the blastula, and in the gastrula a decrease anteriorly and more or less laterally from the dorsal lip of the blastopore. From neurula stages on, removal from the membrane is less difficult, the embryos are less fragile and can be moved about, and dyes penetrate more readily. In open neurula stages reduction progressed from the anterior region of the plate posteriorly and in the plate and folds earlier than laterally and ventrally. In later stages reduction progressed from the head posteriorly and from the dorsal region laterally and ventrally (Child, 1941, p. 157). Data for the

neurula and later stages appeared to be definite and conclusive, but those for gastrulae and earlier stages were regarded as not completely demonstrative.

During the last two years sodium hydrosulphite ($Na_2H_2S_2O_4$) has been found extremely useful in work on dye reduction in other material as a means of decreasing oxygen content of water or dye solution rapidly or slowly to any desired degree. Methylene blue in solution can be reduced at once, and dye reduction can be brought about in a few seconds in living stained material if desired, or oxygen content of the solution containing the material can be somewhat decreased and the preparation sealed for further oxygen decrease and dye reduction by oxygen uptake of the living material. Reduced methylene blue penetrates much more rapidly than oxidized dye; consequently, hydrosulphite is extremely useful in staining the amphibian material. Embryos within the vitelline membrane stain very slowly in solutions of oxidized methylene blue in the concentrations used in these experiments (1/10,000 or less, usually 1/20,000); but, if they are brought into a solution of oxidized dye and the solution is then reduced by hydrosulphite, they stain almost at once. Evidently the reduced dye penetrates much more rapidly than the hydrosulphite and is oxidized in the cells of the embryo. Hydrosulphite is, then, as useful for rapid staining without removal of the vitelline membrane as it is for reduction.

Only a minute amount of hydrosulphite—a few granules, constituting a small fraction of a milligram—is required to reduce completely one to several milliliters of 1/20,000 methylene blue. Dry air is necessary for accurate weighing, and the amounts required are so small that weighing is impracticable. With a little experience the amount required for a given volume and concentration of dye can be very closely approximated, and if too little is used more can be added. If amounts used are only enough or little more than enough to reduce the dye completely, the hydrosulphite is very soon all oxidized; and if the solution remains open to the air reoxidation of the dye may begin within a minute or two in consequence of entrance of oyxgen from the air. In solutions which are 8–10 mm. deep, reoxidation at the deeper levels may not occur for 5 or 10 minutes or more if there is no stirring or agitation. The embryonic material after reduction by hydrosulphite can be reoxidized rapidly or slowly as desired by varying depth of solution and amount of agitation. If the preparation is sealed at once after addition of hydrosulphite, the dye solution, of course, remains reduced, but embryos may remain more or less stained until further oxygen decrease brings about their reduction.

Embryos can be reduced and reoxidized several times without preventing further development. Many have developed into normal animals after these procedures. In the concentrations used, hydrosulphite is not appreciably toxic except with long exposure in sealed preparations. As already noted, embryos oxidize methylene blue in solutions reduced by hydrosulphite, evidently because the dye penetrates and the hydrosulphite does not, or does so only very slowly.

With staining and reduction by means of hydrosulphite, single embryos can be used and reduction and reoxidation repeated within a few minutes. For reduction by oxygen uptake of the embryos after staining, slides with vertical-walled depressions deep enough to allow the embryos to roll about after covering and sealing and enough embryos almost to

fill the depression have been used in most cases. With 20–30 embryos sealed in a depression 15 mm. in diameter and 4–5 mm. deep, differential reduction becomes evident in 1–3 hours at 20°–22° C., according to stage and number of embryos. The differential reduction pattern under these conditions is the same as that observed with hydrosulphite; evidently toxicity of hydrosulphite is not concerned in the normal reduction pattern.

After several repetitions of reduction and reoxidation, evidence of damage to the embryos does appear, either from continued accumulation of dye in the cells or from hydrosulphite. The susceptibility to damaging action is differential and becomes evident in a differential retardation of reduction, the regions which originally reduce most rapidly being most retarded, so that the reduction gradients are more or less obliterated or reversed in direction. But, after some differential retardation of reduction, recovery is apparently possible, for some embryos showing this effect have developed into normal animals, though death usually occurs sooner or later.

Unfortunately, sodium hydrosulphite is less satisfactory for use with Janus green. It will, of course, reduce this dye either to the red, partially reduced, or the colorless, completely reduced form. The red stage, however, is relatively stable and does not usually reoxidize to the blue-green color; but, after reduction to colorless by hydrosulphite, reoxidation to red may occur on increase of oxygen. The oxidized dye penetrates very slowly; and hydrosulphite can be used to reduce it or to decrease oxygen almost to the critical point with further gradual decrease and reduction by oxygen uptake of the embryos in sealed preparation. Janus green has been less used than methylene blue with amphibian material; but, in stages from the gastrula on, the same pattern of differential reduction appears as with methylene blue.

Dye reduction can, of course, be brought about either in the dye solution used for staining or after removal to water or Holtfreter solution. For reduction by hydrosulphite, dye solution is usually preferable because the color change in the solution serves as an indicator of amount of hydrosulphite required. For reduction without hydrosulphite, dye solution—water or Holtfreter solution—may be used; but here also dye solution seems preferable in order to avoid possible loss of dye from the embryos, although such loss apparently does not occur or is extremely slow. Some color is usually still present two weeks or more after staining.

With all the modifications of procedure it is highly important that the embryos be frequently, or almost continuously, moved about during the primary staining and during reduction and reoxidation in order to avoid local differences in dye concentration, hydrosulphite, or oxygen. In open solutions currents from a pipette serve, and in sealed preparations mixing is accomplished by frequent change of position of the slide with respect to gravity. The earlier stages, of course, orient to gravity; and, if left undisturbed, the same region is always in contact with the glass and is consequently likely to stain less rapidly or to reduce more rapidly than its physiological condition alone would determine. Regions about the blastopore are readily observed with the aid of a mirror a short distance below the slide, and embryos sealed on depression slides can be observed from the side by holding the slide vertically. Since the gastrula orients with the blasto-

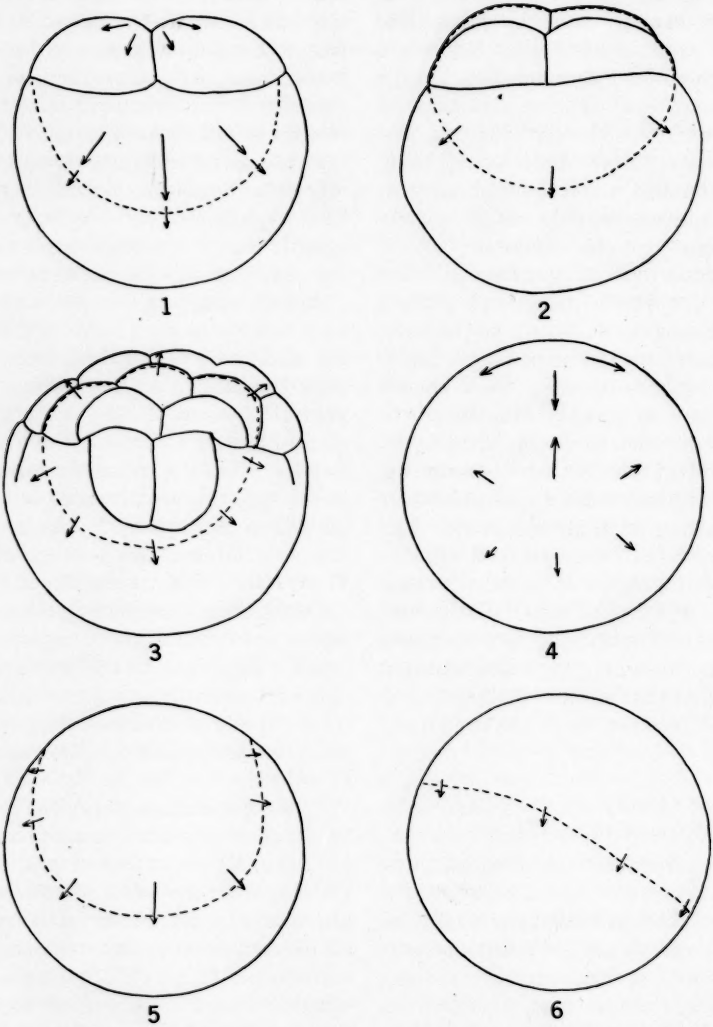
pore somewhat dorsal to the lower pole, it, the dorsal lip, and the dorsal and ventral sides can be seen in these side views.

The figures are diagrammatic attempts to indicate progress, rather than particular stages of the reduction pattern. Arrows indicate general directions of progress of reduction, and lengths of different arrows indicate roughly differences in rate of progress in different regions, longer arrows indicating more rapid and shorter, less rapid, progress. Broken lines inclosing certain regions indicate approximate boundaries of more rapidly reducing regions. Actually, these boundaries are not so sharply defined as the lines indicate; more rapidly reducing regions grade off into less rapidly reducing. The broken lines merely indicate where this gradation is relatively steep. Figures 14, 15, and 16 were outlined with the aid of photographs of embryos made available by Dr. Twitty. Other figures are from sketches of embryos undergoing reduction. Although eggs and embryos of *T. rivularis* are somewhat larger than those of *T. torosus*, both are drawn the same size.

GRADIENT PATTERNS OF CLEAVAGE AND BLASTULA STAGES

In early cleavage stages dye reduction progresses from the apical pole (animal or anterior pole) in the blastomeres; and a region on one side of the undivided portion, presumably the dorsal side, reduces more rapidly than the rest. In *T. torosus* this dorsal (?) reduction becomes evident as early as, or even earlier than, the apical reduction, but the latter is perhaps to some extent obscured by pigment. Figure 1 of *T. torosus* indicates the relatively slower apical reduction. The gradient pattern of reoxidation is the reverse of that of reduction, that is, regions which reduce most rapidly reoxidize least rapidly. In an albino embryo of *T. rivularis* with four apical blastomeres these reduce most rapidly, and below them a region on one side reduces more rapidly than the rest, as in *T. torosus* (Fig. 2). In reoxidation the apical blastomeres and the rapidly reducing side of the embryo reoxidize least rapidly and least completely, and reoxidation progresses in general toward the apical pole. In these albino early cleavage stages the two blastomeres above the region of rapid reduction in the undivided part reduce more rapidly than the other two, suggesting a dorsiventral differential even in the apical region. Figure 2 indicates complete reduction in two of the apical blastomeres and in the region below them, as observed in an albino embryo of *T. rivularis*. Figure 3—a later cleavage stage of albino *T. rivularis*—indicates reduction of apical blastomeres progressing from the side where reduction is more rapid at lower levels. This is also the side on which cleavage furrows progress basipetally most rapidly. Here, too, progress of reoxidation is opposite in direction to that of reduction.

In albino blastula stages of *T. rivularis* two regions of reduction are usually visible in earlier reduction stages, one progressing from the apical pole, the other on one side (Fig. 4). In the further progress of reduction these two regions become continuous (Fig. 5). In cleavage and blastula stages the region about the lower pole and the presumably ventral side reduce much less rapidly than other regions and, except with high concentrations of hydrosulphite, less completely. For example, the region below the broken line in Figure 6, presumably a side view, shows dye color increasing toward the



FIGS. 1–6.—Differential dye reduction in cleavage and blastula stages. Fig. 1, *T. torosus;* Fig. 2, *T. rivularis* albino, early cleavage. Fig. 3, *T. rivularis* albino, later cleavage. Figs. 4 and 5, *T. rivularis* albino blastula; Fig. 4, early, Fig. 5, later, stage of reduction from the presumably dorsal side. Fig. 6 *T. rivularis* albino blastula in presumably lateral view, indicating differential dorsiventral (?) progress of reduction.

lower pole, even when the region above is completely reduced; and even after reoxidation there is usually a graded increase of color toward and below the levels indicated by the broken line.

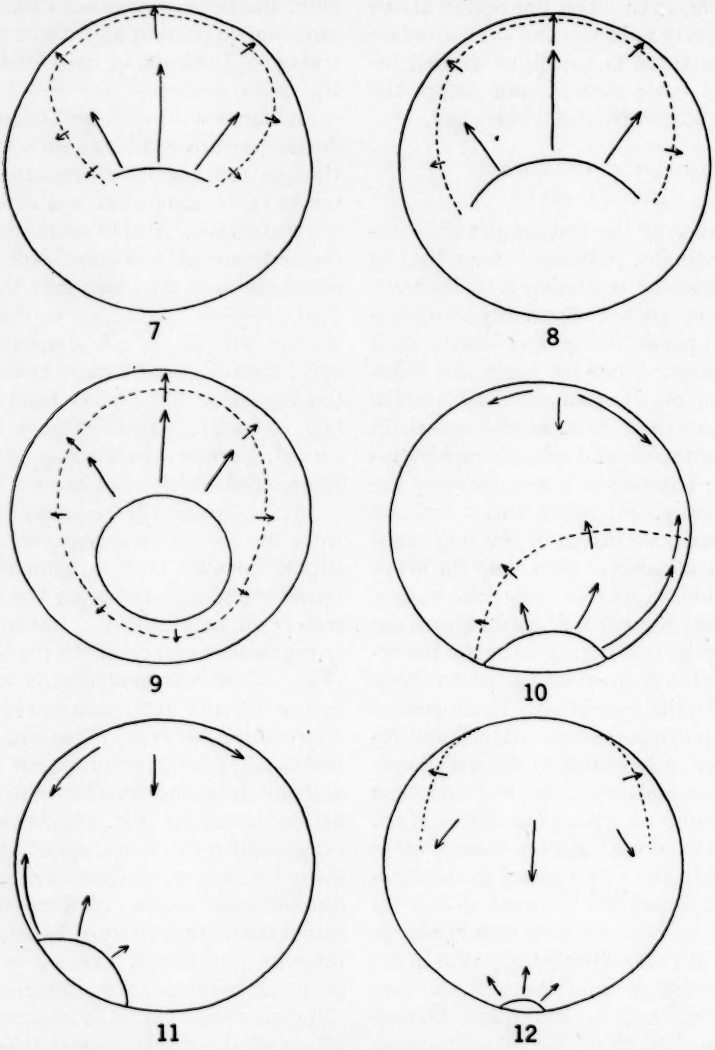
GRADIENT PATTERN OF THE GASTRULA

Gastrulae of the two species show essentially similar patterns, except that in *T. rivularis* the reduction gradient from the anterior pole is apparently more distinct and persists in greater degree than in *T. torosus*. From the beginning of invagination on, dye reduction in *T. torosus* becomes evident, first, at the dorsal lip of the blastopore and spreads rapidly toward the anterior pole and to some extent laterally. Figures 7 and 8 indicate the approximate extent of this more rapidly reducing area as seen from the lower dorsal side in earlier gastrula stages. This rapid progress of reduction from the dorsal lip is not occasionally or uncertainly visible at these stages but has been observed quite consistently in all gastrulae—a hundred or more—stained and reduced, is clearly visible to the naked eye, and has been shown to several members of the Stanford zoölogical staff. Even though the dorsal region stains more deeply than the yolk ventral to the blastopore, the contrast between dorsal lip and yolk as regards reduction is sharp. Progress of reoxidation is opposite in direction to that of reduction, with the dorsal lip reoxidizing last and least. During reoxidation, depth of dye color increases from the dorsal lip in the direction of the arrows on Figures 7 and 8. Lateral boundaries of this region of rapid reduction are surprisingly sharp in many embryos, not, however, in all. When clearly distinguishable, they extend from near the blastopore at an angle of about 45° to the anteroposterior axis. Anteriorly, however, the boundary is not distinct. The same dorsal pattern appears in early gastrulae of *T. rivularis*, most distinctly in the albino embryos.

In stages with circular blastopore the dorsal pattern remains much the same, though the region of rapid reduction seems to be somewhat less extensive in some embryos. Also in some cases an increased rate of reduction extends completely around the blastopore but on the ventral side is limited to the region near the lip (Fig. 9). In a few embryos, however, streaks of more rapid reduction extending somewhat farther from the ventral, or ventrolateral, lip have been observed, perhaps indicating streaks of more rapid cell movement.

In *T. torosus* the reduction gradient from the now definitely anterior pole is slight, appears late as compared with dorsal reduction, and often has not been seen at all, particularly in the more deeply pigmented embryos. In the side view (Fig. 10) dorsal reduction is advanced before anterior reduction is evident. In *T. rivularis* anterior reduction is more distinct. In albino embryos it becomes evident about the same time as reduction at the dorsal lip (Fig. 11); with further progress of reduction, anterior and dorsal areas become continuous, and the whole anterodorsal region appears more reduced than other parts. This region also remains less deeply stained or almost colorless on reoxidation. Anterior reduction progresses ventrally much less rapidly, and the whole ventral side reduces much more slowly and reoxidizes much more rapidly than the dorsal.

These reduction patterns in early and middle gastrula stages have been observed with rapid oxygen decrease and reduction by hydrosulphite, with gradual oxygen decrease by oxygen uptake of the embryos sealed in dye solution entirely



FIGS. 7–12.—Differential reduction in gastrulae. Figs. 7 and 8, *T. torosus*, early gastrulae. Fig. 9, *T. torosus*, later gastrula with narrow zone of rapid reduction surrounding the blastopore. Fig. 10, *T. torosus*, and Fig. 11, *T. rivularis*, advanced gastrulae, indicating the relatively more rapid reduction about the anterior pole in *T. rivularis*. Fig. 12, *T. rivularis;* the faintly indicated anterior neural plate is the most rapidly reducing region and a second, rapidly reducing region appears anterior to the blastopore. The blastopore is drawn somewhat nearer the anterior pole than its actual position in order to show its stage in the same figure as the neural plate.

without hydrosulphite, and with some oxygen decrease by hydrosulphite before sealing and further decrease by oxygen uptake after sealing. Gastrulae have been reduced and reoxidized in dye solution and in water, and the same gastrulae have often been reduced and reoxidized two, three, or even more times both with and without hydrosulphite. The same patterns of reduction and reoxidation appear each time, until, after several repetitions or after deep staining, alteration of patterns indicates injury. Dorsal and anterior reductions are more retarded and may become slower than ventral and posterior, so that the reduction pattern becomes more or less completely reversed, that is, regions originally reducing most rapidly are more susceptible to damage. As far as observed, such embryos may show some further development but die sooner or later. Gastrulae may develop into normal animals after two or three reductions and reoxidations.

A few points concerning later gastrula stages which overlap the beginnings of neural-plate formation are of some interest. In *T. rivularis* the anterior region of the neural plate becomes faintly outlined before the blastopore closes, apparently somewhat earlier in relation to stage of blastopore than in *T. torosus* This difference is perhaps significant, in view of the more rapid reduction about the anterior pole from early stages on and the greater difference in time of development of anterior and posterior neural folds in neurula stages of *T. rivularis* as compared with *T. torosus*. Reduction in this later gastrula, or early neurula, stage of *T. rivularis* shows the pattern indicated in Figure 12. The very faintly indicated anterior region of the neural plate is at this time the most rapidly reducing region of the embryo, and reduction progresses posteriorly from it for a considerable distance. Anterior, and to some extent lateral, to the blastopore there is a small area reducing more rapidly than adjoining regions, though less rapidly than the anterior neural plate. On reoxidation the anterior plate reoxidizes last and least and may remain almost colorless in oxidized dye solution, although primarily it stains no less, or even more, than other parts. This, of course, is the region about the anterior pole, now underlain by chorda-mesoderm. The region anterior to the blastopore also reoxidizes later than regions about it.

Figure 13 shows a stage of *T. torosus* just preceding final closure of the blastopore, viewed from the lower pole. The neural groove is appearing, and an area of relatively rapid reduction extends a considerable distance anteriorly and a short distance laterally from the blastopore. Anteriorly it extends along the longitudinal elevations on each side of the neural groove. The region of early reduction anterior to the blastopore in Figure 12 is a slightly earlier stage of this region, as it appears in *T. rivularis*. The outline of the neural plate becomes faintly distinguishable in *T. torosus* at about this stage, and its anterior region seems to reduce a little more rapidly than posterior levels; but the difference is less distinct than in *T. rivularis* or is uncertain in some embryos at approximately this stage. The deeper pigmentation of much of the *T. torosus* material as compared with that of *T. rivularis* may contribute to the uncertainty, but it appears beyond question that reduction in the anterior part of the very early neural plate of *T. torosus* is somewhat less rapid relatively to more posterior parts than in *T. rivularis*. Outside the neural plate, reduction is much less rapid and progresses in general posteriorly and ventrally.

GRADIENT PATTERN OF THE NEURULA

As already noted, the anteroposterior developmental gradient of the early neural plate of *T. torosus* is somewhat less steep, that is, the outline of the anterior region of the plate becomes distinguishable somewhat less in advance of the posterior region than in the other species. In the early neurula of *T. torosus* the anterior region of the plate reduces early (Fig. 14). In some embryos evidence of a slight gradient from extreme anterior to more posterior levels of this region appears, but more frequently the whole anterior region appears to reduce at about the same time, as indicated in Figure 14. From the anterior region reduction progresses posteriorly; but in these early neurulae the neural groove is developing anteriorly from the blastopore, and reduction progresses anteriorly on each side of it. This reduction begins at the blastopore as early as, or earlier than, in the anterior region of the plate. As far as observed, this reduction gradient from the blastopore does not extend to the anterior end of the fully developed neural groove but disappears anteriorly in the general area of reduction in the anterior plate. In comparable stages of *T. rivularis* the reduction picture is very similar; but the reduction gradient progressing posteriorly appears to be somewhat steeper, and the gradient from the blastopore has not been observed to extend so far anteriorly.

As elevation of neural folds takes place, they reduce rapidly from anterior to posterior levels, and reduction in the floor of the plate becomes evident in the slightly elevated regions lateral and anterior to the anterior end of the neural groove at almost the same time as in the folds, apparently slightly later, with further progress laterally and posteriorly in the floor (Fig. 15). As in the earlier neurula, reduction lateral to the plate is much slower and progresses posteroventrally. Reduction in a still later neurula is indicated in Figure 16. The anterior, most advanced portions of the folds about the still open region reduce first, and reduction progresses posteriorly from them. Even in lightly pigmented embryos the floor of the plate in later neurulae is so deeply pigmented that its reduction relative to other parts remains uncertain. Albino embryos of these stages were not available, but there are certainly no very great differences between the anterior floor and the folds about it.

Progress of reoxidation in the neurulae preceding differential inhibitory effects is opposite in direction to that of reduction, and in oxidized dye solution open to air the wide anterior region and the folds about it (Figs. 14, 15) show less color than other regions, though the primary staining is fully as deep. Differential inhibition becomes evident in more or less complete obliteration or reversal of the reduction and reoxidation patterns.

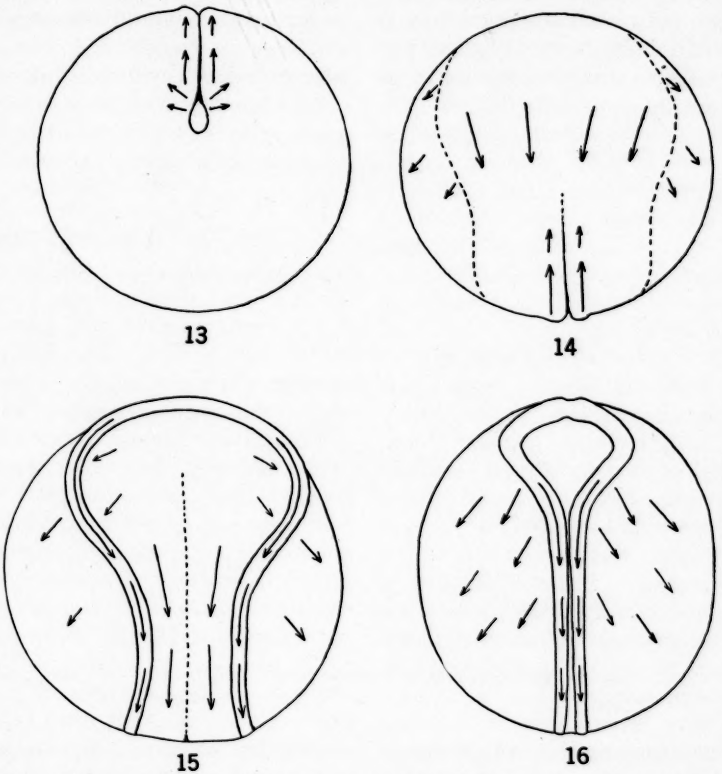
LATER STAGES

The reduction pattern soon after closure of the neural folds is indicated in Figure 17. Reduction progresses posteriorly from the anterior head region, very rapidly along the mid-dorsal region and posteroventrally and much less rapidly in other regions. In a somewhat later stage with tail bud, the reduction pattern is similar, except that the developing tail shows a slight gradient from the tip anteriorly (Fig. 18).

Differential reduction has been observed in still later stages up to animals with well-developed forelegs. Changes in gradient pattern during these stages are chiefly concerned with appearance and development of particular organ systems,

balancers, gills, and legs. In these later stages, as in earlier, reoxidation patterns are the reverse of reduction patterns, and both may be reversed by differential inhibition.

tion shows the earlier stages of the same gradient patterns as reduction in low oxygen but remains incomplete, that is, it shows a gradient pattern in the regions representing the higher levels of the re-



FIGS. 13–16.—*T. torosus*, neurula stages. Fig. 13, the developing neural groove, with reduction progressing anteriorly from the blastopore on each side of it. Fig. 14, early neural plate, with reduction progressing posteriorly from its anterior region and anteriorly from the closed blastopore along the neural groove; in lateral regions, outside the plate, reduction progresses posteroventrally and much less rapidly. Figs. 15 and 16, later neurula stages showing reduction progressing posteriorly in the plate and less rapidly posteroventrally outside it.

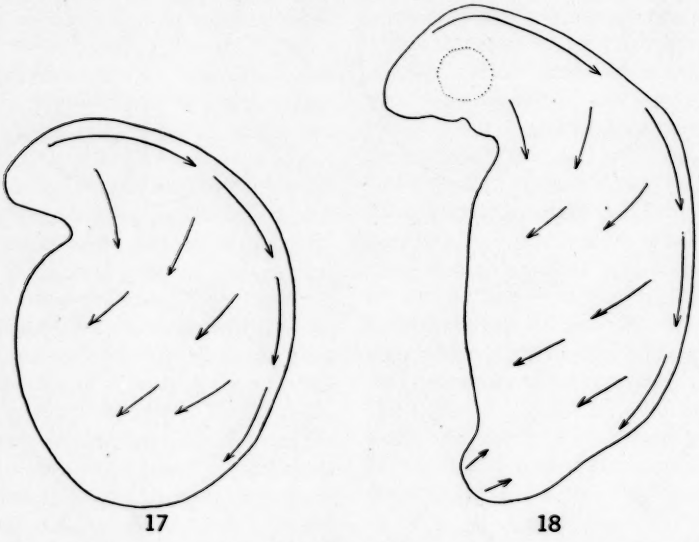
PARTIAL DECOLORATIONS AND RECOLORATIONS

In embryos lightly stained and left in oxidized dye solution with large surface and only a few millimeters deep and open to air, partial differential decoloration usually occurs gradually. This decoloration gradients but is usually inappreciable at the lower levels. For example, the dorsal lips of gastrulae, the anterior polar region of *T. rivularis*, particularly in the albino embryos, the anterior region of the open neural plate, and the head region of later stages undergo more

or less decoloration when left in oxidized dye solution after the rather slight staining ordinarily used in these experiments. From these regions a gradient of increasing color extends.

It has been noted above for the different stages that return of dye color in reoxidation not only shows a gradient pattern opposite in direction to that of reduction but that regions which reduce most rapidly usually remain with less color than other parts. There is not only a difference in time but a difference in degree of recoloration after reduction, although the regions remaining with least color were originally stained as deeply as, or often more deeply than, others. These regions—again the dorsal lip, the anterior polar region of *T. rivularis*, the anterior part of the open neural plate, and the head of later stages—appear as more or less reduced.

Oxygen has not been used to increase further the oxygen content of the dye solution in these cases; but, since some further recoloration has been observed on bubbling air through the solutions, these partial decolorations and recolorations appear to be expressions of differential reductions. Apparently the regions which reduce most rapidly in low oxygen are able to reduce low intracellular dye concentrations slowly and more or less completely in the presence of oxygen content sufficient to oxidize completely dye in solution and to keep such intracellular concentrations more or less completely reduced in oxidized dye solutions. Whether this ability is originally present or is associated with an accelerating effect of methylene blue on respiration, such as has been observed in various organisms, remains uncertain. That there is actual loss of dye or alteration in some other way than reduction, with loss of color, appears improbable, since the embryos



17 18

FIGS. 17 and 18.—The anteroposterior and dorsiventral pattern of differential reduction in later embryonic stages of *Triturus*.

remain continuously in the dye solution in which they were originally stained. However, partial differential decoloration in low concentrations of methylene blue and Janus green after staining with these concentrations has been observed in other material without appearance of the red reduction stage of Janus green (Child, 1942). In any case these partial decolorations and recolorations constitute still further evidence of the presence of definite spatial physiological patterns in these embryos.

REDUCTION OF JANUS GREEN

Janus green has been less used than methylene blue because, as already noted, staining with the oxidized dye is very slow, particularly in the earlier stages, and because complete reoxidation to the blue-green form does not occur under the usual conditions in embryonic stages. Reduction by hydrosulphite in gastrula stages stained with oxidized dye progresses anteriorly and somewhat laterally from the dorsal lip and from the anterior pole, and in neurulae and later stages reduction patterns are like those with methylene blue. With gradual reduction the patterns appear, first, in the red stage, then in the colorless, completely reduced stage, with the same gradient patterns in both. Even though the primary blue-green stain is scarcely visible, the patterns may become brilliantly clear with the progressive change in color to red. With the further reduction to colorless and reoxidation to red, the patterns are visible in progressive decoloration and recoloration, as with methylene blue.

DISCUSSION

The data of differential dye reduction and reoxidation in living embryos and later stages of *Triturus* show beyond question the presence of a definite gradient pattern. As regards reduction, this pattern appears to be an expression of a spatial pattern involving enzyme activity and, as regards reoxidation, of a spatial pattern of oxygen uptake. Moreover, its relation to the spatial pattern of morphogenesis is evident. The occurrence of differential reoxidation in cleavage and blastula stages does not conflict with the fact that at these stages development can continue under anaerobic conditions with the aid of glycolysis (Brachet, 1934; Lennerstrand, 1934), while under aerobic conditions the embryo uses oxygen.

In cleavage and blastula stages reduction is more rapid on one side of the embryo than on the other. If this is the dorsal side, as seems probable, it is evident that dorsal and ventral differ, at least quantitatively, in these early stages. The dorsal region of rapid reduction and slow reoxidation in the gastrula evidently represents the dorsal inductor region. Moreover, the reduction-gradient pattern within this region coincides rather closely with the gradient of inductive capacity from the dorsal lip anteriorly and laterally, and the reoxidation pattern is the reverse of this. The rather close correspondence between the dye-reduction pattern in pregastrula and gastrula stages and the differential-susceptibility pattern of the frog embryo (Bellamy, 1919) is of interest as indicating that both are concerned with aspects of the same general physiological pattern. Reduction and reoxidation patterns of the neurula and later stages appear as further developments of these patterns in the gastrula and earlier stages.

Since the patterns of differential reoxidation are the reverse, as regards direction of progress, of the reduction patterns, they provide a check on the reductions and another line of evidence indi-

cating presence of definite gradient patterns. The partial decolorations and recolorations in the presence of sufficient oxygen to oxidize completely the dye in solution evidently constitute still another expression of the gradient patterns. And, finally, the more or less complete reversal of regional differences after repeated reductions and reoxidations or deep staining or both apparently represents a differential inhibition resulting from regional differences in susceptibility to hydrosulphite or dye or the combined effect of both.

It is possible that differential dye reduction and reoxidation in intact living embryos may be more nearly correct comparative indicators of regional differences in certain features of developmental pattern involving oxidation-reduction activities than measurements of oxygen uptake in small isolated fragments. In the amphibian embryo dye reduction and reoxidation indicate certain activities of living, metabolizing protoplasm independently of yolk content of the cells; but, as already noted, regional differences in yolk content of cells, such as are present in amphibian embryos, may be complicating factors in determining the real significance of regional differences in respiratory rates (Barth, 1939*a*, *b*, 1942; Child, 1941, pp. 153–56). Boell and Nicholas (1940), using the "ultramicrorespirometer" and very small fragments, maintain that oxygen uptake of the dorsal lip is no greater than that of other regions, when correction for yolk is made. Barth (1942), using a micro-Winkler method with several pieces and also with correction for yolk content, finds rate of oxygen uptake distinctly higher in the dorsal lip than ventrally and highest in the animal polar region. Moreover, by comparing oxygen uptake of the whole gastrula with that of the sum of isolated pieces of a gastrula, he finds that cutting the gastrula into six parts has little immediate effect on oxygen uptake. There may be a slight stimulation following isolation, but after 24 hours the rate of oxygen uptake of isolated pieces is less than that of the intact gastrula. He also finds that within certain limits pieces of different size with different surface-volume relations do not differ significantly in rate of oxygen uptake. These findings go far toward answering the question of effects of isolation of pieces on oxygen uptake, but whether regions of higher rate are more affected by isolation than those of lower rate still remains to be determined.

Whether rates of respiration of living, metabolizing protoplasm of the amphibian embryo do or do not differ regionally, it is beyond question that intracellular reduction of methylene blue and Janus green occurs much more rapidly, and reoxidation of these dyes much more slowly, in the dorsal lip than on the ventral side of *Triturus* gastrulae. In fact, at certain stages reduction is more rapid and reoxidation slower in the dorsal lip than in any other region of the embryo, except perhaps the extreme anterior pole in *T. rivularis*. There is primarily an apicobasal (animal-vegetal) dye reduction and reoxidation gradient, but even in early stages, one side, undoubtedly the dorsal side, stands out as a region of more rapid reduction and less rapid reoxidation than the opposite side. Differential dye reoxidation suggests a higher oxygen uptake in regions of less rapid reoxidation, that is, with gradual oxygen increase in the medium following reduction in the embryo, reduced regions of high oxygen uptake may be expected to show reoxidation of dye less rapidly than those using less oxygen. Data on dye reduction and reoxidation are obtained from

intact embryos still capable of further development, except after several repetitions of the reduction-oxidation cycle. The data on oxygen uptake which show no difference between dorsal inductor and ventral regions, some without, and others with corrections for yolk content, were determinations on small fragments of tissue.

It is perhaps scarcely necessary to point out that, while reduction and reoxidation patterns show the existence of certain quantitative regional differences, they give no information concerning presence or absence of other differences. However, in view of their presence from early developmental stages on, and their definite relation of morphogenetic pattern to them, there can be little doubt that the quantitative gradient patterns are fundamental factors of development.

SUMMARY

1. Oxidized methylene blue stains earlier embryonic stages of *Triturus* very slowly, but the dye reduced by sodium hydrosulphite penetrates almost at once and oxidizes to some extent within the embryo, even without removal of the vitelline membrane.

2. After staining, reduction by hydrosulphite or by oxygen uptake of embryos sealed in dye solution and reoxidation after reduction by gradual increase of oxygen show definite gradient patterns characteristic of developmental stage and undergoing definite changes in the course of development. In cleavage and blastula stages the apical or anterior (animal) polar region and one side, presumably dorsal, reduce most rapidly and reoxidize least rapidly; and reduction progresses from, reoxidation toward, these regions.

3. In the gastrula the dorsal inductor region reduces more rapidly and reoxidizes less rapidly than lateral and ventral regions. Within it reduction progresses from the dorsal lip anteriorly and somewhat laterally, and reoxidation in the opposite direction. Reduction also progresses from, and reoxidation toward, the anterior polar region, very distinctly in *T. rivularis*, less distinctly and later in *T. torosus*.

4. In neurula stages reduction in the neural plate progresses in general posteriorly, except for a temporary gradient extending anteriorly some distance from the closing or closed blastopore, as the neural groove develops. Outside the plate, reduction progresses posteroventrally. Reoxidations progress in the opposite directions.

5. After closure of the neural folds, reduction progresses rapidly from the anterior head region along the median dorsal region and much less rapidly posteroventrally in lateral regions. In earlier stages of tail development a short reduction gradient with progress from the tip appears. Reoxidations progress in the opposite directions.

6. After light staining, partial decolorations and incomplete recolorations usually occur in the presence of sufficient oxygen to oxidize completely the dye in solution. The more rapidly reducing regions gradually decolorize or show less recoloration than other parts.

7. Reduction of Janus green to the red, and further to the colorless, stage and reoxidation from colorless to red show the same gradient patterns as methylene blue. Reoxidation of Janus green from red to blue-green does not occur in the embryos under the usual conditions.

8. Embryos are still able to continue normal development after several repetitions of reduction and reoxidation, either with hydrosulphite, if used with care, or

with reduction by oxygen uptake of the embryos in sealed preparations. With continued repetitions of the reduction-reoxidation cycle, differential inhibition occurs sooner or later with more or less complete reversal of reduction and reoxidation patterns, but some further development is still possible.

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EFFECTS OF DRUGS ON THE HEARTS OF ARENICOLA AND LUMBRICUS

(Two figures)

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ACETYLCHOLINE inhibits the hearts of vertebrates and mollusks; it accelerates the hearts of decapod Crustacea, of *Limulus*, and of the grasshopper, *Melanoplus*. In the vertebrate heart and many molluscan hearts the beat is myogenic in origin, whereas in the decapod Crustacea and *Limulus* the pacemaker tissue is nervous. It is of interest to know the effect of acetylcholine upon the hearts of annelids.

MATERIALS AND METHODS

The action of several drugs upon the hearts and pulsating dorsal vessel of *Arenicola cristata* and *Lumbricus terrestris* was observed by counting the beats during *in situ* perfusion. The counting was sometimes facilitated by tapping the rhythm of several vessels on keys connected to signal magnets recording on a kymograph. Actual kymographic tracings were obtained of the beating *Arenicola* hearts both *in situ* and in perfusion chambers. Light, glass-tipped wire levers were used with magnification ratios of from 3.5 to 11.5 times. Tensions on the isolated hearts were from 65 to 100 mgm. Careful adjustment of tension by means of small wire riders was necessary, since with these, as with all other invertebrate hearts, the strength and frequency of beat increases up to a maximum and then declines with increasing tension and rate of flow. In no cases were the vessels cannulated; solutions bathed them from outside.

Both *Arenicola* and *Lumbricus* were first anesthetized with saturated chloretone solution. Then the hearts were exposed, dorsal body wall pinned back to paraffin blocks, and time allowed for complete recovery during continual flow of saline. Recovery required between 30 and 60 minutes in *Arenicola*, a little less in *Lumbricus*. Before removal of the *Arenicola* hearts the ventral vessel was ligated with the loop which served for attachment in the chamber, the heart and part of the lateral gastric vessel were removed, and either a tiny glass hook inserted in the dorsal part of the heart or a ligature placed around the lateral esophageal vessel for connection to a lever.

Sea water proved an excellent physiological saline for *Arenicola*. In the earthworm both frog Ringer and Bruna's fluid (Jordan and van der Feen, 1930) proved very unsatisfactory. Using Maluf's (1940) analyses and freezing-point measurements of coelomic fluid as a basis and varying the Ca/K ratios over a wide range, we prepared an earthworm saline, which maintained a fairly steady beat for the duration of the experiment, which was 3–4 hours. We have not attempted to keep hearts going for longer periods, but in one a good beat was noticed after 12 hours in the flowing saline.

The composition of this earthworm saline is: NaCl, 0.6 gm.; KCl, 0.012 gm; $CaCl_2$ 0.02 gm.; $NaHCO_3$ 0.01 gm. (to pH 7.4); and H_2O 100 ml. The important characteristics are a higher salinity than Bruna's fluid but lower than frog Ringer and a high Ca/K ratio.

CO-ORDINATION OF VESSEL ACTIVITY

The course of circulation in *Arenicola* has been described by Gamble and Ashworth (1898, 1900) and by Ashworth (1904). The blood flows forward in the dorsal vessel, passes on each side by the gastric plexus to the lateral gastric, and thence to the lateral heart, which pumps to the ventral vessel. The area connecting the lateral gastric to the heart always beats before the heart and sometimes beats several times per single heart contraction. This area is essentially the pacemaker. The dorsal vessel, the lateral esophageal, and some nephridial vessels are contractile. The ventral vessel and posterior lateral gastrics are not.

In *Lumbricus* (Johnston, 1903) the blood flows forward in the dorsal vessel and down by the lateral hearts. The upper, narrow part of the heart always contracts before the larger, bulbous portion. The beat shows considerable irregularity, sometimes there are long pauses in the rhythm, and normally the heart may fluctuate between fast and slow rates.

There is no correspondence between the beat of the dorsal vessel and that of the hearts in either worm, although usually the hearts beat faster and more irregularly. There is often no correspondence between the right and the left hearts in *Arenicola*, but in *Lumbricus* the two hearts of a segment tend to beat together, although this is not invariable. Many observers have noted that annelid hearts continue beating when the central nervous system is removed (Stübel, 1909; Aoki, 1930, *et al.*). There may, however, be regulation from the central nervous system (Gaskell, 1919).

EFFECTS OF DRUGS AND SALTS

The effect of acetylcholine without eserinization upon an isolated heart of *Arenicola* is shown in Figure 1, *A*, and the effect upon the rate of beat of a heart and of a dorsal vessel in *Lumbricus* in Figure 2, *A*, *B*. Similar results were obtained in 16 experiments on 10 specimens of *Arenicola* and 29 sets of observations on 21 specimens of *Lumbricus*. These are in addition to a number of preliminary experiments during the establishment of technique on each form. Acetylcholine causes an increase in frequency, a rise in tone, and may stop the hearts and dorsal vessel in systole. Acetylcholine in a concentration of 10^{-8} in sea water causes about a 25 per cent acceleration in *Arenicola;* the threshold lies between 10^{-9} and 10^{-8}. In *Arenicola* the hearts isolated in a chamber were slightly more sensitive than those *in situ*. In *Lumbricus* 2 out of 6 pulsating vessels were accelerated by acetylcholine 10^{-8} in saline and each of 7 treated with acetylcholine 10^{-7}. The beat of the earthworm hearts is so irregular that precise establishment of threshold is difficult. The hearts of *Lumbricus* are slightly more sensitive than the dorsal vessel (Fig. 2, *B*), and near threshold one heart may be accelerated more than the others. In two preparations of *Lumbricus* the ventral nerve cord was removed from segments 4–18 and acceleration observed as in normal preparations.

Eserine, 10^{-4} in sea water, was applied to 3 isolated *Arenicola* hearts. It had a marked accelerating action (25 per cent). After recovery in sea water the hearts were much more sensitive to acetylcholine than before eserinization (Fig. 1, *B*). In one preparation, for ex-

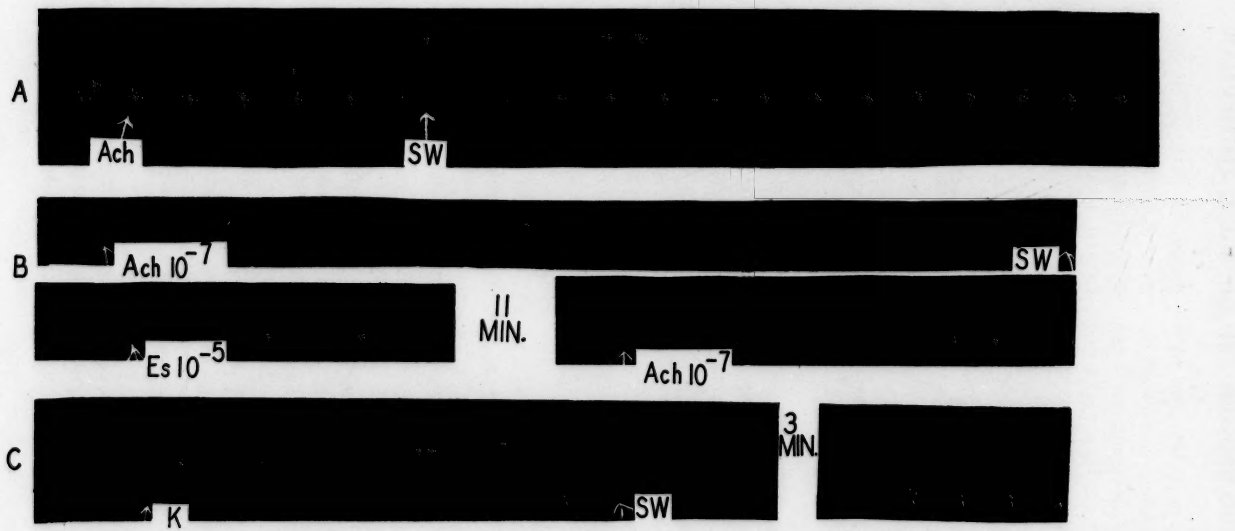

FIG. 1.—Records of beat of isolated hearts of *Arenicola*. *A*, Effect of acetylcholine (approximately 10^{-6}) (*Ach*) and recovery in sea water (*SW*). *B*, Effect of acetylcholine 10^{-7} before and after eserine 10^{-5} (*Es*). Eleven minutes of eserinization omitted. *C*, Effect of increasing potassium in sea water by approximately five times (*K*), and recovery in sea water. Time intervals 30 seconds.

ample, before eserine, acetylcholine 10^{-8} caused a 30 per cent acceleration and no tonus rise, while acetylcholine 10^{-6} accelerated by 90 per cent and caused a rise in tonus but did not stop the heart; after eserinization, acetylcholine 10^{-8} caused a 100 per cent acceleration and a rise in tonus, while acetylcholine 10^{-7} stopped the heart in systole.

Atropine 10^{-4} antagonized acetylcholine in 3 isolated *Arenicola* hearts. Immediate treatment with acetylcholine 10^{-6} after atropinization caused no increase in rate. The effects of atropine were examined on several hearts of each of 8 earthworms. A concentration of 10^{-5} caused very slight inhibition, while atropine 10^{-4} caused marked inhibition. Atropine inhibition was observed in each of 12 vessels; in 6 of these an initial acceleration was also noted. In 10 vessels acetylcholine was applied with atropine after about 10 minutes of atropinization, and there was little or no acceleration (Fig. 2, *C*). When atropine and acetylcholine were applied simultaneously

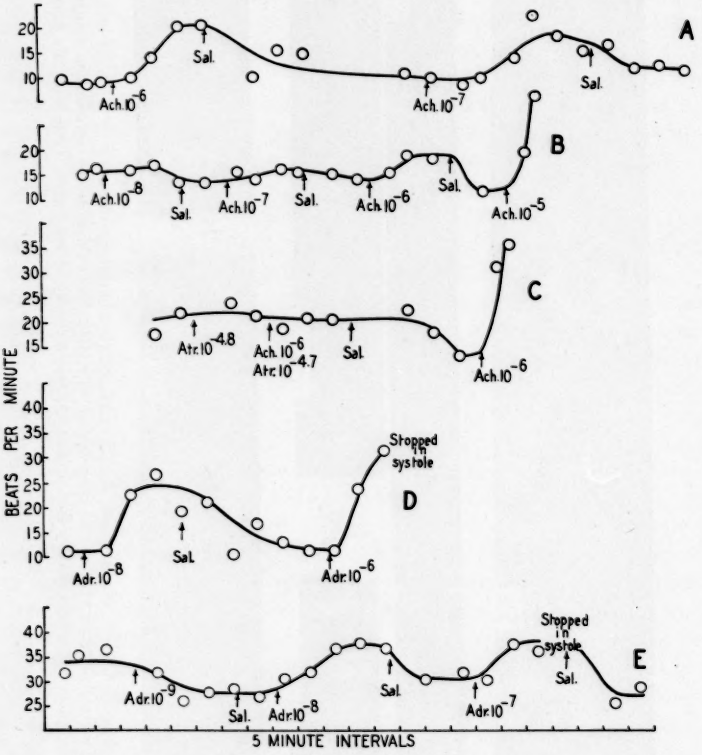

FIG. 2.—Effects of drugs on rate of heartbeat in *Lumbricus*. *A*, Effects of acetylcholine (*Ach.*) 10^{-6} and 10^{-7} and recovery in saline (*Sal.*) on one heart in a worm from which the ventral nerve cord was removed from segments 4–18. *B*, Effect of acetylcholine on dorsal vessel of an earthworm. *C*, Effect of acetylcholine after atropinization (*Atr.*) and after washing out the atropine. *D*, *E*, Effects of adrenalin (*Adr.*) on two earthworm hearts. Each point represents the average of several measurements during each 5-minute interval.

without previous atropinization, acceleration was marked, indicating that acetylcholine acts more rapidly than atropine. Gaskell (1914) noted acceleration with atropine in the leech. Either the leech is different from *Lumbricus*, or else Gaskell saw only the brief initial acceleration found occasionally in *Lumbricus*.

Nicotine was applied to both *in situ* and isolated hearts of *Arenicola* (5 experiments). It acts much like acetylcholine in causing a marked acceleration, a rise in tonus, and cessation in systole. The *Arenicola* hearts are very sensitive to nicotine, and the threshold concentration was 10^{-8}.

Adrenalin yielded inconsistent results with *Arenicola* but very consistent effects with *Lumbricus*. In two preparations of isolated hearts of *Arenicola* in concentration 10^{-5} it caused a very slight acceleration and then stopped the hearts in diastole. This was repeated several times on these hearts. The same effect was obtained on another heart *in situ*. With lower concentrations ($10^{-6.3}$), acceleration was noted in 2 *in situ* preparations on the first few trials, but later application of adrenalin in even higher concentrations had no effect. The results of 13 sets of observations on 6 preparations of *Lumbricus* show that the hearts and dorsal vessel of this worm are accelerated by adrenalin, that the threshold is low (10^{-8}), and that slightly higher concentrations (10^{-7}–10^{-6}) stop the hearts and dorsal vessel in systole (Fig. 2, *D*, *E*). This high sensitivity of the earthworm vessels to adrenalin is of interest in view of the presence of chromaffin cells in the ventral nerve cord (Gaskell, 1914, 1919). Gaskell found adrenalin to accelerate the pulsating vessels of *Hirudo*, but Federighi (1928) obtained no effect on *Nereis*.

Excess potassium (1.5–5×) had the same accelerating action on the *Arenicola* hearts as acetylcholine in 5 experiments on 4 preparations (2 in chamber and 2 *in situ*). Figure 1, *C* shows the effect of increasing the potassium content of the sea water from 0.0104 M to 0.05 M. The heart is more sensitive than the dorsal vessel to increased potassium.

Excess calcium ($2\frac{1}{2}$×) caused slowing and relaxation of 2 *Arenicola* hearts. The effects of potassium and calcium, therefore, are the same as upon the hearts of decapod Crustacea.

DISCUSSION

The experiments indicate that in their pharmacology the hearts and dorsal vessel of *Arenicola* and *Lumbricus* closely resemble the hearts of the decapod Crustacea and *Limulus*. These latter hearts have neurogenic pacemakers. The vertebrate hearts and many of the molluscan hearts which are inhibited by acetylcholine and high potassium are myogenic. In the vertebrates acetylcholine stimulates the nerve cells of the sympathetic ganglia. The heart ganglia of the Crustacea are essentially autonomic ganglia, so that the basic action may be similar in both groups. Much evidence indicates that acetylcholine accelerates neurogenic hearts and inhibits myogenic hearts (Prosser, 1942). On this basis one would predict that the hearts of *Arenicola* and *Lumbricus* are neurogenic.

Nerve cells have been described in the hearts of *Arenicola* by Carlson (1908) and of *Lumbricus* by Stübel (1909). They are not figured in the *Nereis* hearts (Retzius, 1905; and Federighi, 1927, 1928). The nature of possible mediators of nervous acceleration and of inhibition of the annelid hearts has not been investigated.

Some annelid hearts contract by means of a layer of smooth muscle cells (*Lumbricus* shown by Bergh, 1900; and Johnston, 1903; *Arenicola* shown by Ashworth,

1904; *Hirudo* shown by Gaskell, 1914). In other annelids (e.g., *Nereis*) contractions are due to endothelium and Rouget-like cells (Retzius, 1905; Federighi, 1928). The pharmacology of the two types of heart may well be different.

Gaskell (1914, 1919) observed acceleration of the leech pulsating vessels by atropine and adrenalin. He injected muscarine and noted a slowing and inhibition in diastole. From this statement Pantin (1935) suggested that the annelid hearts resemble those of vertebrates. We have not tried muscarine, but it is possible that the leech differs from *Arenicola* and *Lumbricus*. The action of muscarine may not be critical, since Nukada (1917) found it to slow and inhibit in diastole the neurogenic heart of *Limulus*, which is accelerated by acetylcholine. Davenport (1942), on the other hand, obtained acceleration with muscarine on the heart of the crab, *Cancer*.

SUMMARY

1. Beats of *Arenicola* and *Lumbricus* hearts and dorsal vessels were counted *in situ*, and beats of *Arenicola* hearts were recorded kymographically.

2. A physiological perfusion saline for use with the earthworm has been developed.

3. Acetylcholine accelerates, raises tone, and stops these hearts in systole. The threshold is between 10^{-9} and 10^{-8} for *Arenicola* and between 10^{-8} and 10^{-7} for *Lumbricus*. Nicotine acts similarly to acetylcholine on *Arenicola*.

4. Eserine potentiates, and atropine antagonizes, the acetylcholine action. Atropine alone slows the heart of *Lumbricus*.

5. Excess potassium accelerates, and calcium inhibits, the *Arenicola* heart.

6. Adrenalin has an accelerating action on *Lumbricus* hearts (threshold 10^{-8}). Its effect on *Arenicola* is uncertain.

7. In their pharmacology the hearts and dorsal vessels of *Arenicola* and *Lumbricus* more closely resemble the neurogenic hearts of decapod Crustacea and *Limulus* than the myogenic hearts of the vertebrates and mollusks.

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THE ACTION OF VARIOUS TYPES OF ELECTRIC FIELDS ON THE RELATIVE VISCOSITY OF THE PLASMAGEL OF AMOEBA PROTEUS[1]

(Four figures)

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IN SPITE of the extensive use of electric current in physiological experimentation, a complete picture of the action of this stimulating agent on the whole protoplasmic colloid is lacking. Moreover, the data which have been obtained, with possibly two exceptions, are qualitative rather than quantitative in nature.

Brücke (1862), working with human leucocytes; Kühne (1864) with *Tradescantia virginica;* Chifflot and Gautier (1905) with *Cosmarium;* and Bayliss (1920) with *Tradescantia* and *Amoeba* (*sp.*) concluded, on the basis of their results by the method of Brownian movement, that electric current increases the viscosity of the protoplasm of these cells. Employing the centrifuge technique, Bersa and Weber (1922) and Angerer (1939), studying, respectively, the stalk (epicotyl) of the bean plant, *Phaseolus multiflorus*, and the unfertilized egg of the sea urchin, *Arbacia punctulata*, arrived at a similar conclusion, though the latter investigator found a transient decrease, prior to the ultimate increase, in viscosity.

In the light of our knowledge of the physical structure of protoplasm, there are two physiologically important areas of the cytoplasm which demand investigation. These areas are the stiff, cortical layer—the so-called "plasmagel"—and the more fluid, medullary portion—the so-called "plasmasol." Angerer (1939) interested himself specifically in the effects of various electric fields on the medullary portion of the sea urchin's egg, i.e., the plasmasol. The stiff, cortical area (plasmagel), as found in the unfertilized egg of the sea urchin, is of such dimension as to preclude any determination of the relative viscosity by the centrifuge method. Therefore, the more favorable material, *Amoeba proteus*, is resorted to at this time for a study of the effect of electric current on this cytoplasmic layer.

METHOD

The electric fields explored at various current densities were direct (D.C.) and alternating (A.C.) electric currents and discharges from electrical condensers. The material studied was *A. proteus;* the specific cytoplasmic layer investigated was the plasmagel. Amoebae were originally purchased from the Carolina Biological Supply Company; they were cul-

[1] This work was aided by a grant-in-aid from the society of the Sigma Xi to C. A. Angerer, and the experiments on direct current were performed while at the University of Pennsylvania.

[2] We wish to express our thanks to Professor W. J. Kostir, of the Department of Zoölogy and Entomology, Ohio State University, for placing the facilities of his laboratory at our disposal during a portion of the work.

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tured, and normal amoebae were obtained according to the technique described elsewhere (Angerer, 1942). We are indebted to Mr. E. V. Fowler, of the Department of Zoölogy and Entomology, Ohio State University, for supplying amoebae in early experiments with alternating current. Since the electrolytic concentration of the culture fluid proved to be important in obtaining reproducible results, all solutions were prepared with water distilled through a silica still for the D.C. experiments and triply distilled through a Pyrex-glass still for the A.C. work. From 3 to 5 days prior to actual experimentation, amoebae were transferred to Chalkley's (1930) fluid. This solution was used exclusively thereafter for all experimental work. Throughout the D.C. work and during the final experiments with A.C. all culture fluids were maintained between pH 6.6 and pH 6.8, with HCl when necessary. Temperatures of room and of culture fluids were maintained between 17° and 24° C., although the temperature during the course of any one experiment was relatively constant.

The viscosity of the protoplasm was determined by the centrifuge method. An Emerson hand centrifuge, which developed a centrifugal force of 2,474 times gravity when the handle was turned at the rate of one revolution per 2 seconds, was employed in these experiments. The end-point used, in comparing the viscosity of the experimentally treated with that of the "control" or normal amoebae, was the time in seconds necessary for a constantly applied centrifugal force to move the intracellular crystals into one hemisphere of the more or less spherical cell. The time in seconds necessary to reach this end-point in more than 80, but less than 100, per cent of the amoebae centrifuged is termed the "centrifuging time" or "relative viscosity" (ordinates of all figures). The centrifuging tests were determined at intervals of 10 centrifuging units, but, in those experiments which revealed low values for the viscosity, tests were made at intervals of 5 units.

Amoebae were subjected to various current densities in a celluloid trough, the sides of which were perfectly milled and thus parallel to the lines of current flow. The current was applied through a $Zn/ZnSO_4$/Chalkley's-fluid-in-agar system in the D.C. experiments, while a $Cu/CuSO_4$ junction was used in the A.C. work. The half-cells were in circuit with a reversing switch, a milliammeter, and the necessary electrical resistance. The current source was the regular 110-volt (A.C. = 60 cycles) service line running into the laboratory. The agar bridges were cut to fill the ends of the trough completely and were sealed in place by means of hot agar–Chalkley's solution.

When a prolific culture showed amoebae to be normal, i.e., gave a centrifuging value of 160 arbitrary units, it was employed experimentally as follows: At least 10 amoebae were placed in the desired electrical field of known current density and for various known periods of time; several determinations were made for any one point; these always included a "negative" point, i.e., one of an immediately lower value than that designated as "positive." Preliminary experiments were run for various current densities, although ultimately the following current densities were selected for detailed study: 2.5×10^{-3}, 10×10^{-3}, 20×10^{-3}, and 30×10^{-3} amperes/cm.^{2} for D.C.; and 2.3×10^{-3}, 5.4×10^{-3}, and 7×10^{-3} amperes/cm.^{2} for A.C. The fluctuation in current density during the course of an experiment did not exceed 5 per cent.

In the case of the D.C. work, experiments were performed in order to obtain from 8 to 10 curves for each of the current densities listed (see legend, Fig. 1), while in the A.C. work more than 19 experiments (i.e., complete curves) were distributed among the various current densities studied. This procedure entailed the use of at least one culture of amoebae for the development of any one curve at any specific current density, although, granting that sufficient amoebae remained from a previously used culture, these amoebae were then employed for the study of another curve but at some other current density. Finally, with these data as a basis, all the curves representative of the current densities for both the D.C. and the A.C. studies were repeated twice, respectively, with two prolific cultures: one set of data thus obtained for the D.C. work is presented in Figure 1, while another set for the A.C. work is given in Figure 2. This procedure affords a comparison of the effect of the respective current densities under relatively constant experimental conditions.

Those cultures which had previously supplied amoebae for the data representative of the action of current densities of 10×10^{-3} and 5.4×10^{-3} amperes/cm.^{2} for D.C. and A.C., respectively, were used to study the rate of recovery of the relative viscosity from the experimentally induced minima characteristic of these current densities (see Fig. 4; compare with Figs. 1 and 2).

RESULTS

1. *The effect of various current densities applied continuously for varying intervals of time.* (*a*) *D.C. experiments.*—(See Fig. 1 and legend.) The effect of the action of D.C. on the centrifuging values (ordinates) of the plasmagel of *A. proteus* for various intervals of time in seconds (abscissae) as represented in Figure 1 shows that for a current density of 2.5×10^{-3} amperes/cm.^{2} there was no apparent change in the centrifuging time during the first 90 seconds of current flow. It was found on subjecting amoebae during the next 30 seconds to this current density that there was a progressive decrease in the centrifuging time from 160 to 130 units, after which a new minimal value was maintained for longer times of exposure to this current density. After 30 minutes the experiments were discontinued. When the current density was increased to 10×10^{-3} amperes/cm^2., the results differed as follows: The latent period was found to be shorter (8 seconds); the centrifuging times were decreased to a greater degree (decrement of 50 per cent with a current duration of 50 seconds); and the minimal value at which the viscosity leveled was lower than when a current density of smaller value was employed. There was a complete absence of any detectable latent period when current densities of 20×10^{-3} and 30×10^{-3} amperes/cm.^{2}, respectively, were used. The centrifuging values decreased with 3 and 1 seconds of current flow to 50 and 25 arbitrary units, respectively, and leveled at the minimum characteristic of the current density in question. It was found with continued application of these current densities that anodal breakdown ultimately ensued. The time rate of the appearance of anodal breakdown is a function of the current density employed, other variables remaining constant. The solid characters (Fig. 1) signify that more than 50 per cent of the amoebae failed to undergo anodal breakdown, while the outlined characters on the same curve show that over 50 per cent of the cells had undergone anodal breakdown. Roughly, all cells sub-

jected to these current densities (20 × 10^{-3} and 30 × 10^{-3} amperes/cm.^{2}) were cytolyzed after approximately 60 and 30 seconds' exposure, respectively, to continuous current flow. The broken-line portion of the curve for current density 20 × 10^{-3} amperes/cm.^{2} (Fig. 1) signifies that during the tests for exposures between 60 and 90 seconds some amoebae failed to undergo the characteristic anodal breakdown. This failure of all the cells to cytolyze is represented by the broken-line portion of the curve in question.

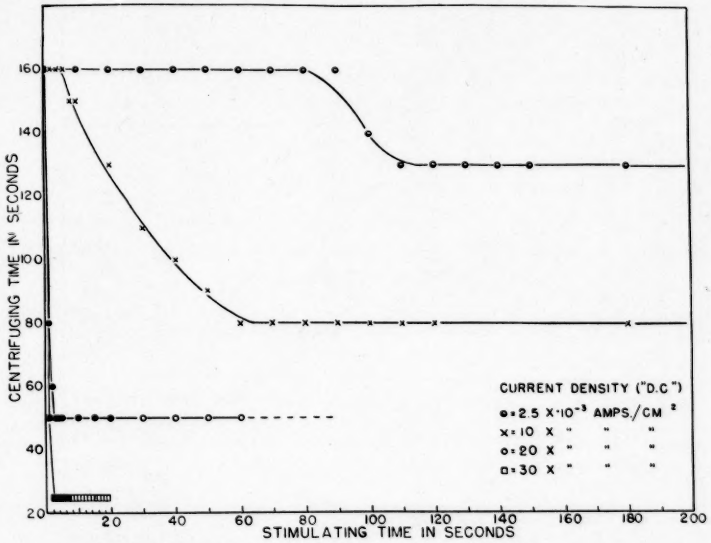

FIG. 1.—Centrifuging time or relative viscosity (*ordinates*) of the plasmagel of *A. proteus* following exposure to direct electric current in seconds (*abscissae*). Current densities employed as noted. When outline and solid characters appear as points on the same curve, the former signify that over 50 per cent of the cells have become cytolyzed. An interval of 10 seconds elapsed between the cessation of exposure to electric current and incipient centrifugation. Temperature 19°–20° C.; pH 6.6–6.8.

The results representing the action of D.C. on the plasmagel of normal *A. proteus* (Fig. 1) are readily reproducible, provided cognizance is taken of the electrolytic concentration of the culture fluid. When the concentration of electrolytes of the Chalkley's fluid was decreased—i.e., the fluid approached the composition of "silica-distilled" water—the previously described experimental results were obtained more rapidly. This observation is interpreted on the well-known principle that the flow of electric current follows the line of least resistance and that more current passed through the amoebae than in the surrounding medium of lower electrolytic content.

b) A.C. experiments.—(See Fig. 2 and legend.) The results for the action of various A.C. current densities on the viscosity of the plasmagel of *A. proteus* are given in Figure 2. A comparison of these data with Figure 1 reveals that the general shape of the curves for the two sets of data is quite similar. The differences between the A.C. and the D.C. data are

as follows: (1) A.C. curves fail to show the so-called "latent period" characteristic of D.C. when exposed to relatively low current densities; (2) A.C. electric fields are more efficacious in lowering the viscosity of the plasmagel both in time and in degree than are D.C. electric fields; (3) when the respective centrifuging values at which the viscosity levels for any specific current density (ordinates) are plotted against these same current densities (abscissae), it is found that the D.C. data fall along a curve of exponential order, while the curve for the A.C. data is represented by a straight line (Fig. 3). It will be seen from this figure that for a given current density A.C. is more effective than D.C. in decreasing the viscosity of the plasmagel.

Experiments carried out with 7×10^{-3} amperes/cm.^{2} in a pH range of 6.1–6.5 and at temperatures between 13.5° and 21° C. gave similar results, except that the curve did not always exhibit a zero slope prior to the death of 50 per cent of the animals.

2. *The time rate of recovery of centrifuging values* for A.C. and D.C. current densities from their respective experimental-

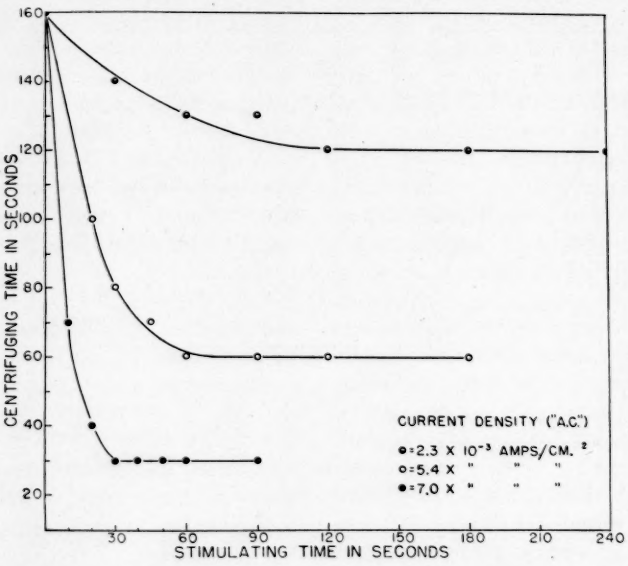

FIG. 2.—Centrifuging time or relative viscosity (*ordinates*) of the plasmagel of *A. proteus* following exposure to alternating electric current in seconds (*abscissae*). Current densities employed as noted. An interval of 10–20 (average, 15) seconds elapsed between the cessation of exposure to electric current and incipient centrifugation. Temperature 21°–22° C.; pH 6.7.

ly induced minima to those values characteristic for normal *A. proteus* is shown in Figure 4. (*a*) *Direct current*, when applied to amoebae obtained from the same culture as that from which the data for Figure 1 were derived, produced after an exposure of 2 minutes at 10×10^{-3} amperes/cm.^{2} a decrease in viscosity from the original (normal) value of 160 arbitrary units to the experimentally induced minimum of 80 units (ordinates). Thereafter, permitting varying intervals of time to elapse from the cessation of ex-

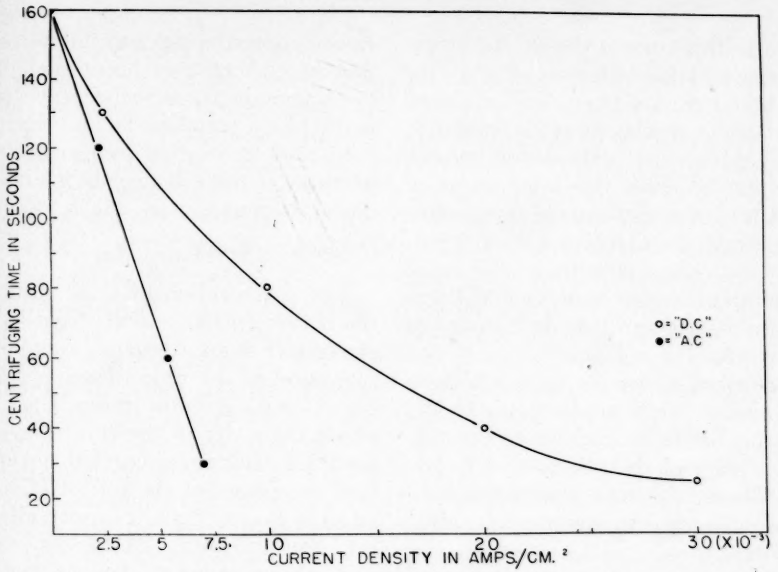

FIG. 3.—Data derived from Figs. 1 and 2, showing the specific values in centrifuging time at which the viscosity leveled for a specific current density (*ordinates*) plotted as a function of that current density (*abscissae*).

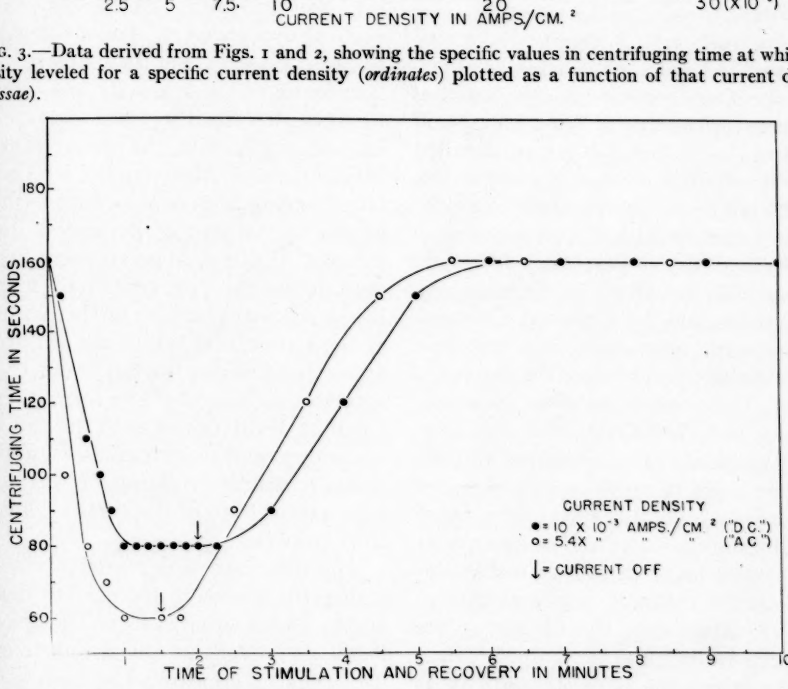

FIG. 4.—Centrifuging time or relative viscosity (*ordinates*) of the plasmagel of *A. proteus*, showing time of exposure to the specific current density noted and the subsequent recovery in relative viscosity from the cessation of this exposure to incipient centrifugation. Amoebae were from same cultures and other experimental variables were the same as those found, respectively, in Figs. 1. and 2.

posure to this current density to incipient centrifugation (abscissae, Fig. 4), the data fall along a sigmoid-shaped curve. The viscosity regains its original value in about 4 minutes. (*b*) *Alternating current*, when studied from the same point of view but with an exposure of 1.5 minutes to a current density of 5.4×10^{-3} amperes/cm.^{2}, gives essentially the same relationship between recovery in the centrifuging values and time in minutes as observed for D.C. (Fig. 4).

3. *Discharges from electrical condensers* of 2 and 4 microfarads, respectively, were tested as to their effects on the relative viscosity of the plasmagel (*A. proteus*). The results were essentially similar, i.e., a decrease in centrifuging values was noted.

4. *Locomotion* of *A. proteus* in various A.C. and D.C. electrical fields has been studied. Our results are in essential agreement with those of Mast (1931) and Hahnert (1932) and will not be detailed here. We shall mention only some observations made on the reactions of amoebae subjected to an A.C. current density of $5.0–5.8 \times 10^{-3}$ amperes/cm.^{2} for 30–60 seconds. At the above current density pseudopodia may be retracted and locomotion may cease during current flow and for a short period after the current is broken. In a great many cases, however, cessation of locomotion occurs only during a portion of the current flow, and the amoebae begin to move again before the current is broken. As previously pointed out, stimulation for 30 and 60 seconds at the above density causes a marked decrease in the viscosity of the plasmagel (Fig. 2). Moreover, the viscosity does not reach its original level until 3 or 4 minutes after the stimulation. It is therefore evident that amoeboid movement can take place when the viscosity of the plasmagel is greatly decreased. Locomotion during and following the flow of current may be normal. However, some of the amoebae have abnormally broad pseudopodia and change direction of locomotion frequently. Small portions of the cell may be pinched off during stimulation (cf. Mast, 1931).

DISCUSSION

The presentation of these results on the plasmagel (*A. proteus*) together with the former study (Angerer, 1939) on the plasmasol of the unfertilized egg of the sea urchin, *Arbacia punctulata*, completes the study of the effect of various electrical fields on the cortical and medullary cytoplasm of the cell. The general effect of electric current (direct, alternating, and condenser discharges) on the cortical cytoplasm of *Amoeba proteus* is to cause a decrease in its viscosity, i.e., a liquefaction. These results are contrary to those described in the literature (see introduction), with the possible exception of those of Mast (1931), who postulates varying degrees of localized solation at the anodal end of *A. proteus* during galvanotropism. Angerer (1939), however, found the "conventional" increase in the relative viscosity of the plasmasol of the unfertilized egg of the sea urchin. In the light of this work it seems reasonable to conclude that the former results listed in the literature as to the "effect of electric current on protoplasm" were essentially studies treating of the medullary, rather than of the cortical, layer of the cytoplasm.

The time rate of decrease in viscosity is directly related to the current density employed for either A.C. or D.C. electric fields. However, for any specific current density A.C. appears to be more potent in bringing about this liquefaction. Likewise, there is a specific value at which the experimentally lowered viscosity levels,

and this varies directly with the current density used. This relation between the rate of decrease in viscosity and the value at which the viscosity levels off is not specific for electrical stimulation but is typical of all stimulating agents which have been studied on a moment-to-moment basis. It is a striking fact that there is no linear decrease ad infinitum in the viscosity when the electric stress is continued indefinitely with time for any current density employed within the biokinetic range. For any specific current density there is apparently a specific minimal value to which the viscosity will decrease and there will level, regardless of the duration of the time of exposure to the specific electric field in question. This was shown by exposing *A. proteus* to the two lowest D.C. densities for 30 minutes and is similarly true for those A.C. electric fields explored. What this means in terms of protoplasmic structure is open to no clear-cut interpretation at this time.

A reversible decrease in the viscosity of the plasmagel is apparently the typical reaction to all these stimulating agents when administered within the biokinetic range. A decrease in the viscosity of the plasmagel (*A. proteus*) was noted for ultra-violet radiations (Heilbrunn and Daugherty, 1933), mechanical agitation, electric current, and thermal stimulation (Angerer, 1936, 1937, 1940). Heilbrunn pointed out the similarity between the results produced by stimulating agents and those obtained when the plasmagel was subjected to decalcifying agents (citrates and oxalates). Hence, on the basis of this and other evidence he concluded that the cortical layer released bound calcium on stimulation and that this was reflected in the subsequent decrease in the viscosity of this layer (for a detailed discussion of his theory see Heilbrunn's review of 1940). While the cortical layer (plasmagel) shows an increased fluidity when subjected to these agents, the main mass of the cytoplasm (plasmasol) shows, ultimately, an increase in protoplasmic viscosity with these same stimulating agents, viz., ultra-violet radiations (Heilbrunn and Young, 1930; Heilbrunn and Daugherty, 1933), mechanical agitation, and electric current (Angerer, 1936, 1939), as studied in both the sea-urchin egg and *Amoeba*.

The appearance of the latent period for relatively low D.C. and not for the corresponding A.C. curves might be explained as correlated with the greater efficacy of the latter field. Were weaker A.C. current densities explored, a similar latency might become apparent.

Angerer (1936) was impressed with the labile nature of the cortical cytoplasm and its tendency to conform to the criteria for thixotropic gels of inanimate nature. While a rapid isothermic breakdown (fluidity) in gel structure under pressure is one of the criteria for thixotropy, equally important is the restitution of its structure to the former degree of rigidity upon removal of the impinging stress. This criterion is adhered to in the case of the cortical cytoplasm of *A. proteus*.

SUMMARY

1. The centrifuge method was used to determine the relative viscosity of the plasmagel of *A. proteus* after being exposed to either D.C. or A.C. electric fields or discharges from electric condensers for known periods of time and at various known current densities.

2. *Amoeba proteus* exposed continuously to relatively low D.C. densities (Fig. 1) shows a pronounced latent period followed by a decrease in the viscosity to a minimal value, at which it remains

on further exposure to the current density in question.

3. Relatively stronger current densities (Fig. 1) produce a corresponding shortening or complete absence of any latent period and a proportionately greater decrease in viscosity to a minimal value at which it levels. Continuous exposure to the specific current density ends ultimately in the characteristic anodal breakdown of the amoebae.

4. Alternating-current densities (Fig. 2) produce essentially comparable results, i.e., a decrease in viscosity of the plasmagel. The following exceptions are noted: (*a*) A.C. fails to show the latent period characteristic for exposures to relatively low D.C. current densities; (*b*) A.C. electric fields are more potent in lowering the viscosity of the plasmagel as regards both time and degree than are D.C. fields; (*c*) when the respective centrifuging times at which the viscosity levels for the specific current density in question are plotted against these current densities, it is found (Fig. 3) that the D.C. data fall along an exponential curve, while the A.C. data show a straight-line relation.

5. Following exposure to D.C. or A.C. (Fig. 4) at a current density and duration which will cause a decrease in viscosity from a value of 160 units to 80 and 60 units, respectively, the original value of 160 units is regained in approximately 4 minutes.

6. Amoeboid movement may occur when the viscosity of the plasmagel has been markedly decreased by exposure to alternating current.

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POTENTIAL-DIFFERENCE MEASUREMENTS ON CHAOS CHAOS[1,2,3]

(Two figures)

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CONSIDERABLE attention has been given to the measurement of potential differences manifested by single-celled animals and plants, chiefly with a view toward an elucidation of the structure and function of the cell membrane. The large algal cells have received most attention (Osterhout, 1931), but protozoan cells have been studied by some workers (Ettisch and Péterfi, 1925; Ettisch, 1928; Telkes, 1931; Kamada, 1934; Buchthal and Péterfi, 1936).

A study of the giant amoeba, *Chaos chaos* (Schaeffer, 1936), is herein reported. The measurements described are solely those between a pair of electrodes arranged so that one contacts the organism, while the second contacts the environing medium. The measurements are hence directly comparable to most of the above-mentioned animal cell studies. *C. chaos* is particularly favorable material, for its large size simplifies the difficulties attendant upon the insertion of microelectrodes.

MATERIAL AND METHODS

Chaos chaos (Type A; Schaeffer, 1937) was cultured in 0.001 per cent NaCl. This dilute NaCl solution is hereinafter referred to as "saline" and is prepared by dissolving C.P. NaCl in twice-distilled water (second distillation out of Pyrex). Large numbers of *Paramecium caudatum*, cultured separately in the same saline solution (with boiled wheat grains added, in accordance with the suggestion of Hyman [1931]), were added to the amoeba cultures to serve as food organisms (Schaeffer, 1937). Watch glasses (2 inches in diameter) were employed as culture vessels. The amoebae were transferred into fresh saline every 5 or 6 days.

The apparatus which was employed in the measurements has been described (Wolfson, 1941, 1942). It consisted of a combination of a vacuum-tube voltmeter, a micromanipulator, an inverted microscope, and silver-silver chloride microelectrodes. The voltmeter has a maximum sensitivity of 45 microvolts/millimeter and an input impedance of at least 5×10^8 ohms. Saline in the microelectrode serves as electrolyte, and, since it can be compressed by a screw-activated rubber diaphragm, the microelectrode is capable of functioning as a micropipette. The openings in the microelectrode tips measured 10–40 μ in diameter.

Specimens of *C. chaos* were regarded as normal if they showed (1) active streaming movements, (2) characteristic "shape," and (3) good "color"—three criteria which can be employed readily after some experience with the organism.

[1] Abstract published (Anat. Rec., Suppl., 78:59, 1940).

[2] A condensation of sections of a dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, in the Department of Zoölogy, Graduate School, University of Kansas, May, 1939.

[3] The writer is indebted to Drs. H. H. Lane, W. J. Baumgartner, C. V. Kent, and J. D. Stranathan for many kindly offices.

Such normal specimens were selected from the various available cultures, ranging up to 5 days in age. Each specimen was then transferred through four successive baths of saline and was then pipetted into a drop of saline previously placed on the "stage" of the inverted microscope (cf. Wolfson, 1942). If this stage (which is merely a cover slip) is clean, the organisms soon attach themselves to it and are then much more easily contacted by the microelectrode tip than is the case when the organism floats in the medium (cf. Chambers and Kopac, 1937, p. 72). By this procedure, then, the organism is transferred from its saline culture medium into a fresh drop of saline and is thus subjected to a minimal environmental change. In the process, however, the cell comes to be environed by a known medium, for unknown chemical bodies (i.e., metabolites) which are present in its culture medium are largely eliminated from its environment by the washing process.

Immersion of the organism in a medium of known composition at the time of measurement is, obviously, an objective of paramount importance. Accordingly, all precautions militating toward this objective were observed. Thus new, chemically clean watch glasses were used as containers for the saline in which the amoebae were washed, a separate pipette was used in transferring an organism between successive baths, the cover slip serving as stage was scrupulously cleaned, etc.

Two microelectrodes were employed, one mounted on each control unit of the micromanipulator. After the laved specimen had been placed in the drop of saline on the microscope stage, the two microelectrode tips were lowered into the liquid, quite close to the specimen in the field of the microscope. One of the two microtips was then brought into contact with the organism.

The organisms were at room temperature during the time of measurement. Room temperature varied between 24° and 29° C.

The length of time during which the potential-difference measurement was continued was recorded for each specimen.

TERMINOLOGY

The electrode which contacts the saline environing the cell is herein designated as "external," and the electrode which is in direct contact with the cell is designated as "internal."

The internal electrode made contact of one of two types, as follows: (*A*) The microelectrode tip presses upon and indents the cell (Fig. 1*a*); and (*B*) a column of protoplasm is gently sucked into the microelectrode tip, and the ectoplasm is then ruptured (Figs. 1*b* and 2).[4]

OBSERVATIONS

Chaos chaos manifests an immediate response when a microelectrode tip is brought into contact with it. Protoplasmic streaming ceases abruptly, as it has been observed to do in various other forms in response to mechanical shock (cf. Heilbrunn, 1937, p. 242). Concomitantly, there is to be observed a quick flow of endoplasmic crystals, which centers toward the microtip. Very shortly, however (1 minute or less), protoplasmic streaming is resumed at a normal, or even an enhanced, rate, and the organism appears entirely typical. Almost

[4] Rupture of the ectoplasm is readily effected through the application of alternate negative and positive pressures upon the protoplasmic column, through the liquid electrolyte in the electrode. Such negative and positive pressures can be applied simply by backward and forward movement of a rubber diaphragm, which is built into the electrode (for construction of electrode, see Wolfson, 1941).

without exception, the amoebae free themselves from contact with the microelectrode tip. If the contact is of Type A, in which case the microtip indents the cell more or less deeply, the amoeba merely flows away, sometimes leaving a few strands of ectoplasm adhering to the microtip. If the contact is of Type B, in which case a column of the organism's protoplasm is imprisoned within the microtip, the amoeba pinches off this protoplasmic column, generally within 5–10 minutes, effecting such disjunction at the mouth of the microtip. In either case any potential difference obtaining prior to such escape is immediately dissipated.

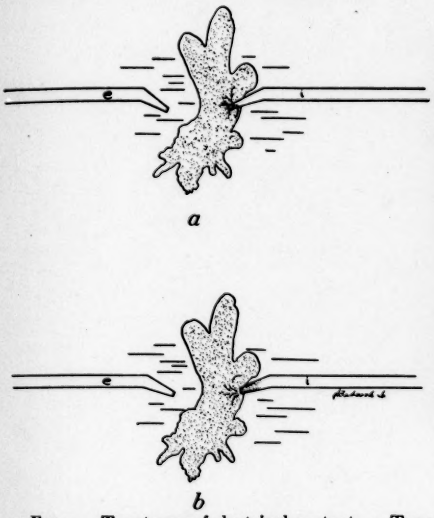

FIG. 1.—Two types of electrical contact. *a*, Type A contact, showing tip of internal microelectrode pressing upon (and indenting) *Chaos chaos; b*, Type B contact, showing protoplasmic column drawn into tip of internal microelectrode and rupture of the column's ectoplasm; *i*, tip of internal microelectrode; *e*, tip of external microelectrode.

It is, of course, most important to know the nature of the junction established between (1) the electrolyte of the internal electrode and (2) the cell. When Type A contact obtains, microscopic observation fails to reveal the nature of the junction which exists, other than that the microtip presses upon and indents (or even impales) the cell. Most importantly, it is impossible to observe with the microscope whether or not the microtip has pierced the ectoplasm and allowed the electrolyte of the internal electrode to come into direct contact with the endoplasm. This technical obstacle, which is recognized by Ettisch and Péterfi (1925, p. 463), has been described by Chambers (1922, p. 189) in the following passage:

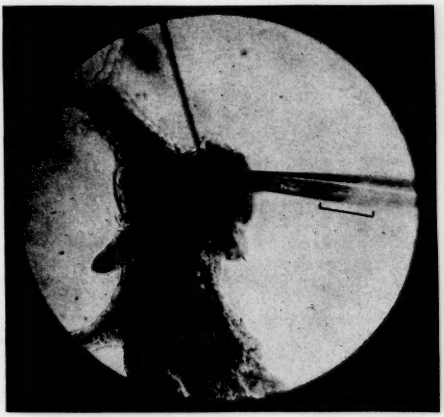

FIG. 2.—Photomicrograph of specimen of *Chaos chaos* on which Type B contact has been established. Note protoplasmic column drawn up into tip of internal microelectrode. Site of the ectoplasmic rupture is indicated by bracket. The rupture is not in focus because the microelectrode tip enters the drop of saline from above and at an angle (as indicated in Fig. 1). External microelectrode not shown. (Indistinct line extending inward from above is shadow of eyepiece pointer.)

> Pushing a pipette, especially a comparatively large one, into an egg cell frequently causes the surface of the cell to become invaginated and thus forms a deep pocket. The tip of the pipette, even if it should finally break through the surface, is apt to be separated from the protoplasm

of the interior by the formation of a new surface film continuous with the original surface of the cell.

It is most important here to note what is pointed out by Ettisch and Péterfi (1925, p. 463), namely, that, if the tip of the internal microelectrode merely indents the cell and fails to pierce the ectoplasm, then the internal electrode is effectively in electrical contact directly with the external electrode through the saline environing the cell and that no potential difference should appear between the two electrodes. When, on the other hand, Type B contact obtains, the relationship between the electrolyte of the internal electrode and the substance of the cell is readily revealed by microscopic scrutinization. For in this case a column of protoplasm is imprisoned within the microelectrode tip (Fig. 2), and rupture[4] of the ectoplasm covering the distal end of this protoplasmic column results in a microscopically observable commingling of endoplasm and electrolyte. Hereby, then, there is established a junction which entails a confluence and intimate union of electrolyte and endoplasm. If the suction being exerted upon the protoplasmic column by the electrolyte be properly adjusted, endoplasmic granules and other formed cytoplasmic bodies are seen to diffuse very slowly into the electrolyte (Figs. 1*b* and 2).

The protoplasm incarcerated in the microelectrode tip during maintenance of Type B contact shows active protoplasmic streaming. If this protoplasmic column be ejected from the microtip, endoplasm quickly flows into it and the ectoplasmic rupture is quickly healed, so that shortly ($\frac{1}{2}$ minute or less) the column looks like a normal, small pseudopod.

Contact with the microtip, be it Type A or Type B, does not wreak any apparent permanent injury upon *C. chaos*. Specimens kept under observation for hours subsequent to a measurement appear entirely normal, as regards both morphological and physiological characteristics.

RESULTS

It was the uniform practice throughout this study first to establish Type A contact on a specimen and then, as soon as the requisite data upon the potential difference manifested with this type of contact had been obtained (1 minute or less), immediately to establish Type B contact.

As soon as the internal electrode comes in contact with *C. chaos*, thereby establishing Type A contact, a potential difference appears between the two electrodes.[5] This potential difference is generally transient, consisting of a voltmeter "throw," which subsides to zero within a few seconds. Occasionally, however, this potential is persistent. The internal electrode is uniformly negative with respect to the external electrode.[6] The maximum potential differences observed range up to 35 millivolts.

When a column of protoplasm is now drawn up into the microelectrode tip, no immediate change in potential difference is observed, i.e., there is no change from the potential-difference value theretofore manifested (with the Type A con-

[5] Prior to the establishment of contact with the organism, a small potential difference obtains between the two electrodes, which are in electrical contact through the saline medium, namely, the "inherent potential difference." This potential difference is balanced out, however, before the internal electrode is brought into contact with the organism. (See Wolfson, 1941, for the voltmeter circuit, which includes a compensating circuit for balancing out the "inherent potential difference.")

[6] That is, the measuring instrument deflects in the same direction as when the internal electrode is connected to the negative pole of a battery and the external electrode is connected to the positive pole.

tact). As soon as the ectoplasm of this protoplasmic column is ruptured, however, and Type B contact thereby established, a pronounced change in the potential difference between the two electrodes unfailingly develops. This alteration in potential difference consists, without exception, of an increase in the potential difference beyond that which had obtained in the given specimen with Type A contact. Thus, if the Type A contact yielded a reading (transient or permanent) showing the internal electrode to be 5 millivolts negative to the external electrode, establishment of Type B contact yielded a reading showing the internal electrode to be perhaps 25 millivolts negative to the external electrode. This Type B potential is never transient but persists as long as the internal electrode is kept in contact with the organism. In this connection it is to be noted that Type B contacts were maintained, in the various specimens, over periods varying from 3 to 11 minutes. With Type B contact, as with Type A, the internal electrode is uniformly negative with respect to the external electrode. Though the Type B potential is persistent, it is practically always inconstant in magnitude, fluctuating at random chiefly within a 5–10 millivolt range but generally attaining momentary extremes outside of this range. Thus, for example, one specimen showed, during the course of a 10-minute measurement, a potential difference which fluctuated chiefly (i.e., during approximately four-fifths of the total duration of the measurement) between 70 and 75 millivolts but which deviated to momentary values as low as 63 and as high as 78 millivolts. Considerable variation in the magnitudes of the potential differences shown by various specimens was observed; thus, one specimen showed a potential difference which fluctuated chiefly between 21 and 23 millivolts, whereas another manifested a potential which fluctuated chiefly between 90 and 95 millivolts.

The potential difference obtaining with Type B contact in any given specimen is independent of the diameters of the openings in the tips of both microelectrodes. This conclusion was reached after numerous microelectrode-tip openings were enlarged by careful fragmentation against the microscope stage without occasioning any alteration in the potential being recorded. That the position of the external electrode does not influence the value of a Type B potential was demonstrated by moving this electrode about in the saline during the course of a measurement. Last, the values obtaining with Type B contact are not dependent upon the length of the protoplasmic column which has been drawn up into the electrode tip, as was demonstrated repeatedly by sucking more protoplasm up into the microelectrode tip during a measurement and noting that no change in the potential then being observed ever ensued.

DISCUSSION

RELATIONSHIPS TO PREVIOUS STUDIES

A critical survey of the reports of previous measurements of the potential differences manifested by single-celled animals (Ettisch, 1928; Telkes, 1931; Kamada, 1934; Buchthal and Péterfi, 1936) reveals that experimental factors of great significance in this type of measurement have not always been accorded adequate investigative attention. Among these factors are (1) the external medium environing the cell, (2) the contact established between the cell and the internal electrode, (3) possible extraneous junction potentials, and (4) the state of well-being of the experimental organism.

In the present study the above-mentioned factors have been given particular consideration. Thus, first, the medium which environs the cell during measurement, namely, saline, is the basic constituent of the medium in which the organism has been cultured, and hence the organism suffers minimal insult when transferred into it. In addition, this medium is known and reproducible. Second, the nature and significance of the contact established between the cell and the internal electrode has been carefully surveyed (as discussed below). Third, an extraneous junction potential which has commonly been overlooked or ignored, namely, a phase-boundary potential between the external electrode and the environing medium, has herein been eliminated, since saline is employed as the medium environing the cell and as the electrolyte in the external electrode. Last, the condition of the specimen has been carefully observed during and after measurement, with the constant result that no manifestations of generalized injury could be detected subsequent to a measurement.

The magnitudes of the steady potentials which were recorded for Type B contact range as high as 95 millivolts. This is a value which greatly exceeds the highest value heretofore reported for steady potentials in single-celled animals, namely, 28.7 millivolts (Kamada, 1934). This divergence of results may be attributable solely to specific differences, since the present report is the first dealing with potentials in *C. chaos*. On the other hand, the high values herein reported are probably attributable, in greater or lesser degree, to the properties of Type B contact, since the previous reports (Ettisch, 1928; Telkes, 1931; Kamada, 1934; and Buchthal and Péterfi, 1936) do not describe the employment of a comparable contact or, indeed, of any contact which unequivocally establishes confluence between the endoplasm of the cell and the electrolyte of the internal electrode.

CRITIQUE OF CONTACTS

The measurements herein described obviously conform in principle to what have been designated by some (e.g., Kamada, 1934) as "membrane-potential" measurements. Electrometric determination of such potential differences which may exist across a cell membrane requires, it must be noted again, that that electrode which is in direct contact with the cell (herein called the "internal electrode") shall have pierced the ectoplasm and be in direct electrical contact with endoplasm. It is important, therefore, to consider in what measure the two types of contact herein employed conform to this requirement.

This prime consideration may be briefly summarized as follows: *Type B contact entails a microscopically observable confluence of* (1) *the electrolyte of the internal electrode and* (2) *the endoplasm. Type A contact may or may not entail such confluence.*

On the basis of its obscureness alone, then, it would seem that contact of the type herein designated as *A* is to be employed with caution, if at all. A further ground for its rejection is the usual transitoriness of the potentials which are registered with it, since it is obvious—to judge from the permanence of the potentials registered with Type B contact—that such transitoriness is imposed by the nature of this contact rather than by any vital processes.

On the other hand, an objection may be urged against the type B contact because it may entail severe injury, and, furthermore, such injury may seriously

influence the magnitudes of the potentials which are observed. The validity of this objection is recognized (cf. Heilbrunn, 1937, p. 304). In so far as the present study is concerned, however, two main lines of evidence make it appear improbable that the observed potentials are attributable to injury effects. These two chief categories of evidence are as follows:

I. Injury entails cellular deterioration or cellular repair. Were the measured potentials dependent upon, or largely determined by, such degenerative or regenerative processes, they would be expected to increase or decrease steadily with the passage of time. It was observed, on the contrary, that the potentials remained fairly constant, as already described.

II. No morphological or physiological evidences of generalized injury were detectable. On the contrary, the following indications of unimpaired cellular physiology were noted:
   a) Locomotor activity, a function of the entire cell, persists during maintenance of the contact, and appears to be entirely normal
   b) Protoplasmic streaming (apparent cessation of which often betokens fatal injury [Heilbrunn, 1937, p. 238]) invariably persists throughout the entire cell and within the protoplasmic column, which is incarcerated by the microelectrode tip
   c) The protoplasmic column may be ejected from the microtip; hereupon, the ectoplasm quickly heals, the protoplasmic column rapidly fills out with cytoplasm, and soon (½ minute or less) the column takes on the appearance of a normal, small pseudopod

In conclusion it may be stated that the magnitudes and durability of the potentials manifested by *C. chaos* are importantly conditioned by the nature of the contact established between the cell and the internal electrode. This conclusion, predictable on a priori considerations, has hitherto been taken into account most effectively by investigators of plant-cell potentials (Osterhout, 1931).

INTERPRETATION OF POTENTIALS

Results on the measurement of a potential difference which appears between electrodes accurately located with respect to *C. chaos* are reported herein. Since these measurements are not contributory of evidence on the question of the ultimate source of biological potentials, this topic will not be entered upon (cf. Bishop, 1941, for a recent, critical review). It may appropriately be pointed out, however, that a simple interpretation of the potentials measured in *C. chaos* by the use of Type B contact regards them as the manifestations of the accompanying electrochemical chain. In this chain the potentials indicated as *b*, *c*, and *d* alone could be of significance, since the two potentials indicated as *a* cancel (being equal in magnitude and opposing in sign), and that potential indicated as *e* is an extraneous junction potential which has been reduced to zero (by employing the same solution as electrolyte of the external electrode and as medium environing the organism). The chain then resolves itself into three phase-boundary potentials, *b*, *c*, and *d*,

−Ag, AgCl	0.001% NaCl (of internal electrode)		Endoplasm of *C. chaos*		Ectoplasm of *C. chaos*		0.001% NaCl (environing *C. chaos*)		0.001% NaCl (of external electrode)		AgCl, Ag+
	a		*b*		*c*		*d*		*e*		*a*

any one or two of which may be zero. Fluctuations in the potentials manifested by such a chain are attributable to variations in b, c, and d, due to unknown processes occurring at phase-boundaries, in the regions between boundaries, or at boundaries and in regions simultaneously.

The potentials manifested with Type A contact consist, for the most part, of a single, short voltmeter "throw," though a small fraction of these potentials are persistent. In either case, whether transitory or persistent, they may well be attributable to alteration in the structure of the phase-boundary at the mouth of the microtip of the internal electrode and be in no special sense attributable to contact of the internal electrode with a living organism.

SUMMARY

Measurements of potential differences manifested by the giant amoeba, *C. chaos*, are herein reported. The measurements are solely those between one electrode which is in direct contact with the cell and a second electrode which is in contact with the 0.001 per cent NaCl in which the organism is immersed.

The electrode which is in direct contact with the cell (internal electrode) can be caused to establish one of two types of contacts, herein designated as *A* and *B*. The potentials observed with these two types of contacts are distinctive, particularly as regards magnitude and permanency. The Type A contact is regarded as altogether untrustworthy, but the Type B contact is regarded as contributory to valid results.

The magnitudes of the potentials manifested when Type B contact is employed are large compared with the values previously reported for such potentials in single-celled animals. It is postulated that this result is attributable either to the specific characteristics of the organism employed in the present study or to the use of Type B contact or to both of these factors.

Though the potentials observed with Type B contact can be viewed as the manifestations of an electrochemical chain which is diagrammed, this view is not offered as an interpretation of the ultimate source of the potentials.

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ELECTRICAL ASPECTS OF A DIURNAL RHYTHM IN THE EYE OF DYTISCUS FASCIVENTRIS[1]

(Three figures)

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INHERENT diurnal rhythms are of considerable interest to the zoölogist because of their frequent occurrence among animals and because of the physiological mechanism implied by these periodic changes. The older literature is reviewed by Welsh (1938). More recently it has been demonstrated that there are diurnal changes in the electrical response of the compound eye of certain beetles (Jahn and Crescitelli, 1940).

The purpose of the present paper is to describe diurnal changes in the electroretinogram[2] of the predaceous diving beetle, *Dytiscus fasciventris*, and to point out that the sensitivity and the electrical oscillations from the eye also undergo diurnal changes.

MATERIAL AND METHODS

Prior to use as experimental material, specimens of *D. fasciventris*[3] were kept in aquariums in the laboratory several weeks under conditions simulating those of the natural environment.

The method of recording the potentials from the eye was similar to that described previously (Jahn and Crescitelli, 1938). The electrical leads were from the corneal surface of the eyes. One silver:silver chloride electrode was in a reservoir of physiological salt solution, which in turn was connected to a solution-filled chamber about the eye by means of a small glass siphon. The front of this eye chamber was transparent and permitted illumination of the eye. The other electrode was connected to the opposite eye, which was prepared in the same manner, except that the front of the eye chamber was made opaque to light. This permitted the study of the potential which developed across the two eyes when one was illuminated. Intensities of the stimulating light are stated as decimal fractions of a unit intensity of about 10,000 foot-candles. The potential change was fed into a condenser-coupled, high-gain amplifier operating under two time constants—2.9 seconds for the slow potentials and 0.1 second for the high-frequency oscillations. The amplifier actuated a cathode-ray oscillograph, the beam of which was photographed on continuously moving sensitized paper.

The animals were prepared as indicated above and placed in a glass-windowed chamber, which was screened from all light except that of the stimulus.

[1] Aided by a grant from the Rockefeller Foundation for work in cellular physiology.

[2] This word has been coined in connection with the electrical response of the vertebrate eye and is defined as "the wave form of the potential change which develops across the illuminated retina" (Kohlrausch, 1931). The use of this term to describe the electrical response of the compound eye seems justified, since it is certain that this response is also closely associated with the photosensory elements. The wave form of the insect electroretinogram is most easily described by using the terminology developed for vertebrates (*a*-, *b*-, *c*-, and *d*-waves), although it is not assumed that the waves are necessarily identical in nature to those from vertebrate eyes.

[3] The authors wish to thank Professor H. E. Jaques, of Iowa Wesleyan College, for the identification of these beetles.

The animals were thus kept in constant darkness, except for the experimental illumination, during the entire period of investigation. The experiments on oscillations and on visual functions were performed at times when the animal presumably was at the height of the day and night phases of its diurnal rhythm. No more than two extensive experiments were performed on any one animal. The humidity was constant, and the temperature was 25° ± 2° C.

RESULTS

I. DIURNAL CHANGES IN THE ELECTRORETINOGRAM OF THE *Dytiscus* COMPOUND EYE

The diurnal changes in the electroretinogram of the compound eyes of *D. fasciventris* are different from those of other beetles previously reported (Jahn and Crescitelli, 1940).

A. *Responses of the dark-adapted eye to a brief flash of unit intensity.*—The typical response obtained to flashes of 17 milliseconds' duration and of unit intensity, during the morning and early afternoon, is a rapid deflection upward, indicating a condition of positivity of the illuminated with respect to the nonilluminated eye. This deflection will be referred to as an *a*-wave (Fig. 1, and record A, Fig. 2). This *a*-wave is followed by a small negative wave or by none at all. When the electroretinogram in response to a brief flash of unit intensity has these characteristics, the eye is arbitrarily classified as being in the day phase. This type of response is usually obtained from 7:30 A.M. until late afternoon.

The electrical response to the same stimulus during the late afternoon and evening consists primarily of a sharp deflection downward, the *b*-wave, followed by the slow, terminal *c*-wave, indicating a condition of negativity of the illuminated with respect to the nonilluminated eye. The *b*-wave may be preceded by a small *a*-spike, as is illustrated in Figure 1 and in records B and C of Figure 2. Both the *b*- and *c*-waves are always present in the response, but the magnitude and duration of the *c*-wave are quite variable. When the electroretinogram has these characteristics, the eye is classified as being in the night phase.

Since the response changes from one phase to another twice every twenty-four hours, it is possible to obtain responses

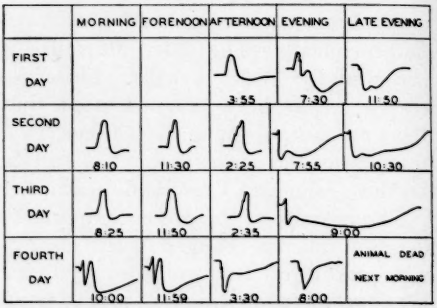

FIG. 1.—Rhythm protocol of a typical *Dytiscus* beetle. The chart illustrates the electroretinograms elicited by brief flashes of high intensity at approximately regular intervals during the entire experimental period. Note that the rhythm tends to persist until death of the animal, which occurred on the morning of the fifth day.

which are transitional forms between the two types just described. These transitional forms often attain considerable complexity and may consist of deflections of about equal magnitude in both positive and negative directions (records D, E, and F, Fig. 2, and second response of the first day in Fig. 1). In 2 out of 20 cases the animals consistently gave a diphasic response (records E and F, Fig. 2) during the night phase and the typical *a*-wave during the day. The reasons for these apparently aberrant responses remain unknown.

To determine the persistence of the

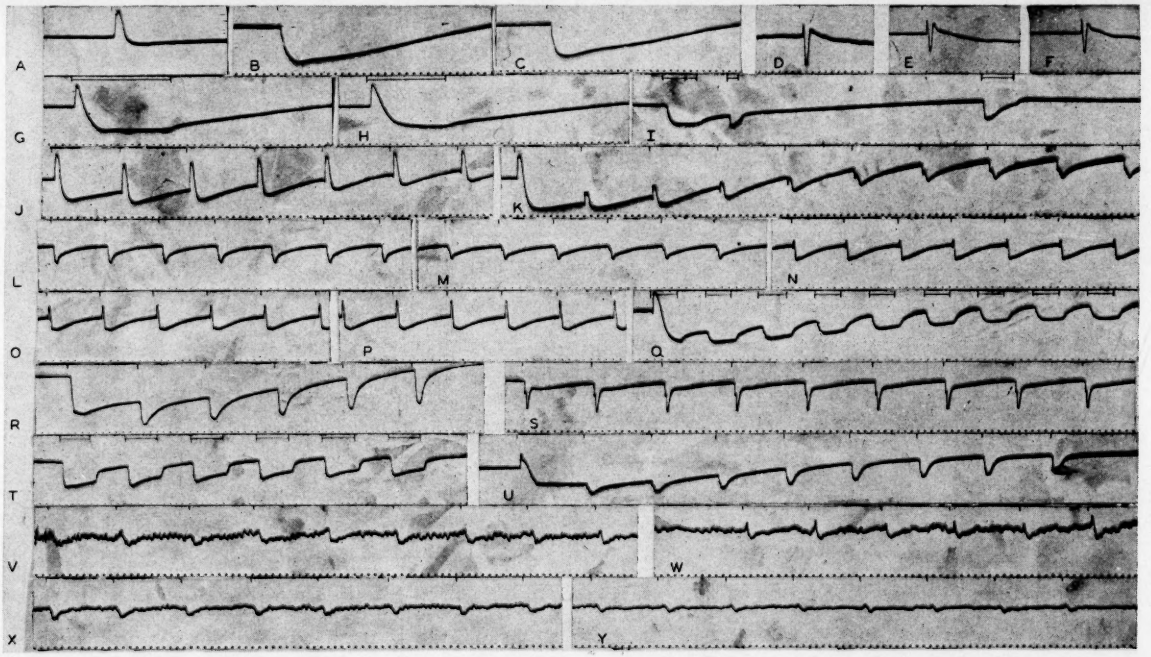

FIG. 2.—Typical responses from the compound eyes of beetles. The records shown are typical responses selected from the great number which were recorded. All the records of Figs. 2 and 3 were taken with the recording paper moving at the same speed. All the records of Fig. 3 were taken with a time constant of 2.9 seconds, and only the responses of records V, W, X, and Y were taken at maximum gain. Records V and X were taken at 5×10^{-4} unit intensity, and records W and Y were taken at 5×10^{-7} unit intensity. The distance between the dots of the time record indicates 100 milliseconds. The stimulus signal is indicated along the upper edge of each record. Refer to text for further details.

diurnal rhythm under the condition of constant darkness imposed upon the beetles from the beginning of an experiment until death, the histories of 20 animals were followed by means of periodic test flashes. The results from 1 typical case are illustrated in Figure 1. It may be seen in this case, more conspicuously in others not presented, that the duration of night phase tends to increase with the time the animal is in darkness. The day phase, however, has never entirely disappeared, except shortly before the death of the animal.

B. *Responses of the dark-adapted eye to longer flashes of unit intensity.*—The electroretinograms obtained from the eye during the day phase in response to flashes varying in exposure from 80 milliseconds to 1 second consist of an *a*-wave followed by a negative wave (probably *b* and *c*). The magnitude and duration of this negative wave increase with the duration of exposure (records G and H, Fig. 2), but the positive *a*-wave remains constant. The night-phase electroretinogram, in response to stimuli of greater duration, apparently consists of only *b*- and *c*-waves (record I, Fig. 2), which may be followed by an off-effect if the exposure is greater than $\frac{1}{2}$ second.

II. THE EFFECT OF LIGHT ADAPTATION UPON THE DAY- AND NIGHT-PHASE ELECTRORETINOGRAMS

A. *Modification of the day-phase response.*—When the beetle eye in the day phase is subjected to repetitive stimuli of 17 milliseconds at a rate of 1 per second, the type of the response depends upon the intensity of the light. When the intensity is only 0.01, the *a*-wave becomes slightly smaller but tends to persist for a long time (record J, Fig. 2). When the eye is stimulated repeatedly with light of unit intensity, however, the *a*-wave disappears as a positive deflection, and the response consists of a *b*-wave by the fourth or fifth stimulus (record K, Fig. 2). In this case there is apparently a direct transformation of the *a*-wave into a notch on the *b*-wave. Records K–P, Figure 3, are parts of a continuous series of responses to repetitive stimuli and demonstrate the changes which take place during light adaptation. The responses of record L show the *a*-wave persisting as a notch on the *b*-wave about midway between the base line and the apex. In record M, taken 3 minutes after the onset of stimulation, this notch on the *b*-wave can be seen to have risen, and in record N, taken 4 minutes after the onset of stimulation, the notch has developed into a small *a*-wave. Records O and P, taken 5 and 6 minutes, respectively, after the onset of stimulation, show the increase in magnitude of the *a*-wave and simultaneous decline of the *b*-wave. This *a*-wave persists with further light adaptation but is markedly decreased when the exposure of the repetitive stimuli is increased. As the duration of the stimulus is increased from 17 milliseconds to 500, the *a*-wave of the light-adapted eye decreases in magnitude and disappears before the exposure reaches 500 milliseconds (record Q, Fig. 2). In this respect it behaves in the same manner as that of the dark-adapted eye. It seems as if the *a*-waves of the light- and dark-adapted eyes may result from the same physiological process which, because of an accommodation phenomenon, results in first an increase and then a decrease in the *a*-wave. The present importance lies not in this relation, however, but, namely, in that the night-phase eye, which exhibits no such phenomenon when treated in a like manner,[4] is, at

[4] A response similar to the primary *a*-wave is also obtained from the dark-adapted night-phase eye

least in this respect, functionally different from the day-phase eye.

B. *Modification of the night-phase response.*—The modification of the typical night-phase response by light adaptation is relatively slight compared to the effect on the response of the day-phase eye. When the eye is repeatedly stimulated with light of 0.2 unit intensity, each stimulus of 17 milliseconds' duration, the apparent size of the *b*-wave decreases after the first flash and then increases (record R, Fig. 2). This may be caused by a decrease in magnitude and duration of the *c*-wave. When the stimulating light is of unit intensity, the wave form is different, the magnitude of the *b*-wave becomes constant, and the magnitude and duration of the *c*-wave are decreased (record S, Fig. 2). When the length of the stimulus is increased to $\frac{1}{2}$ second, the wave form is changed only slightly by repetitive stimulation (record T, Fig. 2).

Repetitive stimulation of an eye in the transitional period may result in one of two permanent states: either a permanent *a*-wave, in which case the eye has been, in a sense, forced into the day phase, or a permanent *b*–*c*-wave, in which case the eye has been forced into its night phase. Record U, Figure 2, shows such a transitional eye in which the diphasic response develops into a permanent *b*–*c*-wave.

III. MEASUREMENTS OF SENSITIVITY OF THE DARK-ADAPTED EYE DURING THE DAY AND NIGHT PHASES

The relative sensitivity of the eye during the day and night phases was measured by determining the minimum intensity which would produce a detectable electrical change. For this purpose a series of repetitive one-per-second test flashes was used, and the lowest intensity which would produce a deflection just large enough to be definitely discernible above the base line of the oscillograph with the amplifier operating at maximum gain was taken as the threshold intensity. The animals were first allowed to become maximally dark-adapted. The intensity of the stimulating light was gradually increased from subthreshold value until a definite response was obtained.

Record V, Figure 2, shows a small response of the dark-adapted, day-phase eye; and record W, Figure 2, shows a response of approximately the same magnitude from the same animal during the night phase. The intensity of the light causing the response of the night-phase eye of record W was a thousand times less than that for the day-phase eye. Records X and Y demonstrate the same for another animal, in which the intensity required to elicit the response of record Y was again one thousand times less than that required to elicit the response of record X.

It seems, therefore, that the eye of the *Dytiscus* beetle becomes much more sensitive to light during the night phase of its diurnal rhythm. This indicates that the factors causing the diurnal change in the electroretinogram also affect the eye in other ways.

IV. RELATION OF OSCILLATORY DISCHARGES TO THE DIURNAL CHANGE IN THE ELECTRORETINOGRAM

A. *High-frequency discharges from the day-phase eye.*—When the dark-adapted, day-phase eye is partially light-adapted and the course of light adaptation is followed with the amplifier operating at maximum gain and 0.1 second time con-

(record I, Fig. 3) at intensities of 0.001 unity or less. With higher intensities the wave form is always in the negative direction (records J and O, Fig. 3), regardless of the state of adaptation.

stant, a high-frequency oscillatory discharge sooner or later appears. This discharge, pictured in records U, V, and W, Figure 3, is similar in appearance to the regular light rhythm of grasshoppers and to the light rhythm of moths (Crescitelli and Jahn, 1942). The discharge is quite regular, with an initial frequency of 29 per second. As stimulation proceeds the amplitude of the discharge decreases and the frequency increases (up to 34 per second at the end of record W, Fig. 3). When illumination of the eye is further prolonged, the rhythm finally disappears. There is no oscillatory discharge present when the eye is not illuminated, and there is no oscillatory response to brief (17-millisecond) flashes of light.

B. *High-frequency discharges from the night-phase eye.*—The oscillatory response of the maximally dark-adapted eye in the night phase to a continuous stimulus of unit intensity consists of a rapidly developing high-frequency discharge of considerable magnitude (record G, Fig. 3). This response is well synchronized by the end of 2 seconds and persists until the light is turned off (at the indicated point near the beginning of record H, Fig. 3). The oscillatory discharge may persist as a post-stimulus response or after-discharge. Exposure to unit intensity for 1 minute or more results in disappearance of the response.

An oscillatory discharge is also elicited by a brief flash of unit intensity, as shown in records A through F, Figure 3. Here the first stimulus of 17 milliseconds excited a poorly synchronized after-discharge. Succeeding stimuli, however, elicited oscillations which became progressively more synchronized and exhibited greater amplitude and greater amplitude variation. The amplitude variations apparently result from the interference of two simultaneous discharges of slightly different frequencies. This after-discharge is similar to the discharge which is elicited by a continuous stimulus of light, as seen in record G.

There are several differences between the oscillatory discharges from the day-phase and night-phase eyes. The night-phase oscillations are subject to regular and rapid amplitude changes, whereas the day-phase rhythm declines very slowly in amplitude; the frequency of the night-phase rhythm is quite constant, while that of the day-phase rhythm increases; the night-phase oscillations are present only during the initial stages of light adaptation, but the day-phase rhythm occurs only after several seconds of light adaptation; the night-phase rhythm apparently persists as an after-rhythm, while the day-phase rhythm stops with cessation of illumination. Furthermore, brief flashes cause no oscillations in the day-phase eye, whereas in the night-phase eye they elicit an after-rhythm very similar in appearance to the light rhythm.

C. *Relation of after-discharge to the b-wave.*—It has been repeatedly observed that the after-discharge of the night-phase eye is not elicited by brief stimuli of low intensity. In the intensity range from threshold (intensity which would just produce a response at maximum amplifier gain) to 0.005 unity the electroretinogram consists of an *a*-wave followed by little or no *b*-wave. Even repetitive stimulation at these intensities does not produce a *b*-wave. When the electroretinogram is of this nature, it is never followed by the high-frequency discharge. However, with intensities of 0.005 unity or more, the after-discharge always appears after the first or second flash. Examination of the electroretinogram elicited by repetitive stimuli at 0.005 of unit intensity (record O, Fig. 3)

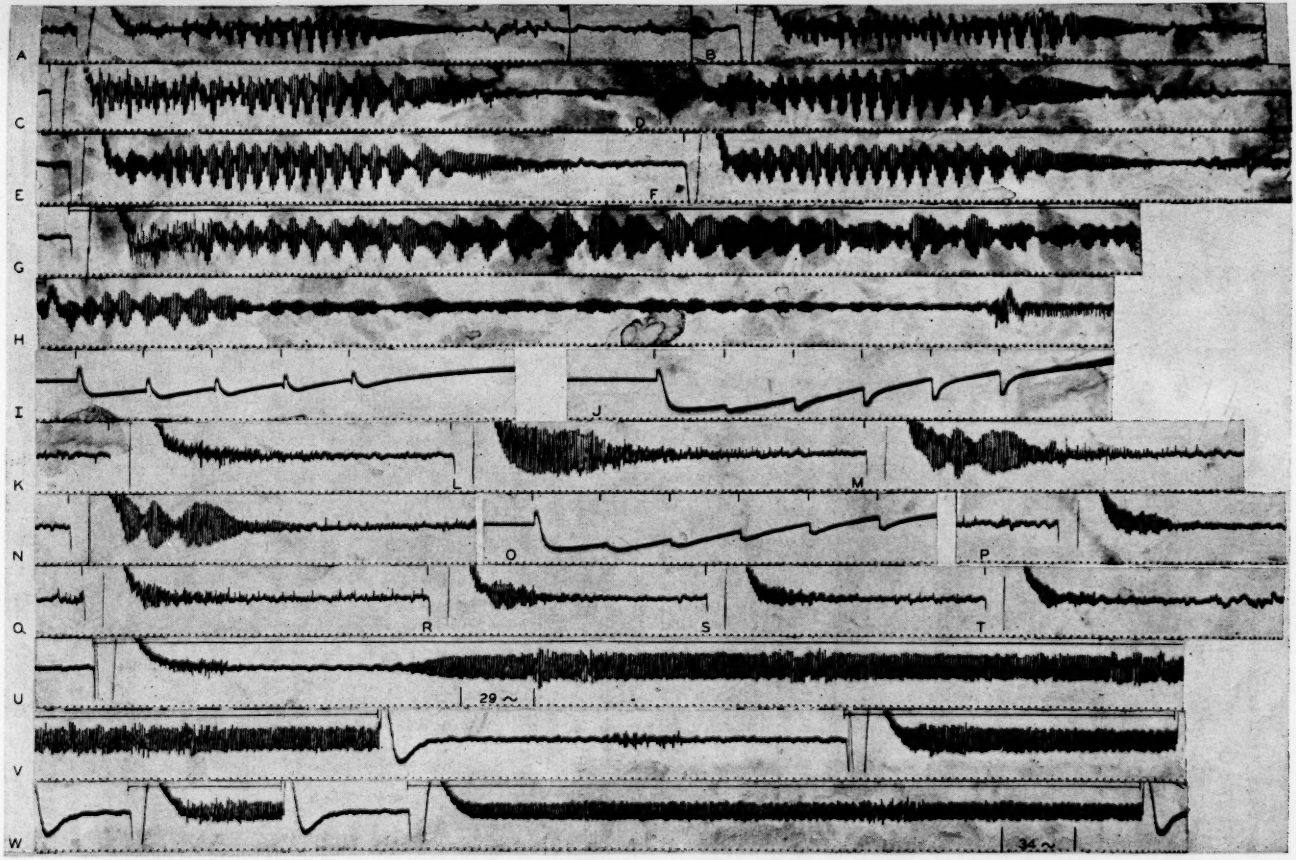

FIG. 3.—Electrical responses from the eye and optic ganglion of *Dytiscus* beetles. All records were taken at maximum gain and 0.1-second time constant, except records I, J, and O, which were taken at a lower gain and 2.9-second time constant. Records A through T are various responses from the night-phase eye. Records U, V, and W are responses from the day-phase eye. Refer to text for further details.

and of 0.01 of unit intensity (record, J Fig. 3) shows that the responses after the first or second flash change into a *b*-wave, which increases in magnitude. Records K, L, M, and N are the responses to successive stimuli of 0.01 unit intensity which were spaced to permit the appearance of the high-frequency discharge (the slow electroretinogram is filtered out), and they show that the after-discharge is quite large and well synchronized at the time of the second flash and undergoes amplitude modulation in response to the third and fourth flashes. Records P, Q, R, S, and T are comparable records for stimuli of 0.005 unit intensity The electroretinograms elicited by stimuli of 0.001 unit intensity are shown in record I and consist of a persistent *a*-wave. At this intensity no after-discharge was observed.

It seems probable, therefore, that the after-discharge, which is so characteristic of the night-phase eye, is functionally related to the *b*-wave of the electroretinogram, which is present in the night-phase response at intensities greater than 0.005 unity. In the day-phase eye this *b*-wave is not present, and the high-frequency discharges do not appear in response to brief stimuli.

DISCUSSION

In view of the facts presented it seems probable that the periodical fluctuations in the electrical responses of *Dytiscus* and other beetles (cf. Jahn and Crescitelli, 1940) are merely an expression of a more fundamental diurnal rhythm involving the physiological state of the eye. This change in the functional state of the eye from the day to the night phase must be of such a nature as to account for (1) the increased sensitivity of the eye in the night phase, (2) the difference in the oscillatory response during the day and night phases, (3) the differences in the wave form of the electroretinogram, (4) the difference in the modification of the electroretinogram with light adaptation, and (5) the diurnal differences in flicker-fusion contours (Jahn and Wulff, 1941*a*).

Since a diurnal rhythm is expressed in the migration of retinal pigment in the eyes of various Malacostraca, one might expect a similar rhythmical migration of pigment to be associated with the diurnal electrical rhythm in the *Dytiscus* eye. In this manner one could explain, in simple terms, the sensitivity differences of the day- and night-phase eyes. However, such a migration does not occur (Jahn and Wulff, 1941*b*), and other differences (electrical oscillations and wave forms of the electroretinogram) indicate that a process more fundamental than pigment migration is involved. It is even possible to consider the day and night phases as two physiologically distinct visual systems.

CONCLUSIONS

1. The electrical response of the eye of *D. fasciventris* undergoes diurnal changes which apparently result from diurnal changes in the physiological state of the eye. These physiological states are referred to as the day and night phases. Evidence for the existence of this rhythmic change of state consists of the following facts: (*a*) the electroretinograms during the day phase and during the night phase are different; (*b*) the modifications of the electroretinogram caused by light adaptation are different during the day and night phases; (*c*) the intensity of light just necessary to cause a perceptible electrical response is about a thousand times greater during the day than during the night phase; (*d*) the oscilla-

tory discharges recorded from the eye are different during the day and night phases.

2. The appearance of oscillatory discharges in the response of the night-phase eye is definitely correlated with the presence of a *b*-wave in the electroretinogram. Both are present at intensities greater than 10 foot-candles, and both are absent at lower intensities.

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SOCIAL BEHAVIOR OF THE WESTERN BANDED GECKO, COLEONYX VARIEGATUS BAIRD[1,2]

(Ten figures)

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ALTHOUGH scattered observations on the habits of various species of Gekkonidae are to be found in the literature, surprisingly little is known of their social behavior. Nocturnal lizards are especially elusive under field conditions, but in the laboratory they can be induced to display apparently characteristic behavior patterns. During the summer of 1939 a large number of the western banded gecko were made available. At the time we were engaged in an extensive analysis of breeding behavior in the American chameleon (manuscript in preparation). It therefore was of great interest to undertake a comparative study of the nocturnal desert-dwelling gecko.

The social behavior of lower vertebrates includes four general activities: (1) aggregation, (2) dominance and territory, (3) sexual behavior, and (4) parental care. Many species are solitary; others show no aggressiveness. Parental service may be entirely absent or limited to the burial of eggs. Nevertheless, these four elements of social organization may be traced through the vertebrate series. The broad, phylogenetic implications of such natural-history data have recently been reviewed by Noble (1939). A somewhat related problem is the determination of the functions of morphological differences between the sexes. The present study was performed in order to provide data on the behavior patterns of *Coleonyx variegatus* and to discover the functions of a remarkable complex of secondary sex characters found in these and many other geckos.

MATERIALS AND METHODS

The animals were maintained within four large adjoining cages, each with floor space of 24×31 inches. Movable glass partitions enabled the observer to combine the areas and inhabitants of adjacent cages. A layer of fine sand several inches deep, strips of green moss, pieces of hollow bark, and water dishes completed the experimental environment.

For convenience of observation, the lizards were kept in dim red light from 9:00 A.M. to 5:00 P.M. and in bright artificial light during the remaining 16 hours. During the period of observation (June 2–August 25, 1939), ordinary room temperature and humidity were adequate. The geckos readily ate mealworms (*Tenebrio*), blowflies (*Calliphora*), and any other insect larva or soft-bodied adult.[3]

[1] This study was completed under the direction of the late Dr. G. K. Noble, curator of the Departments of Herpetology and Experimental Biology of the American Museum of Natural History, whose unfailing interest, inspiration, and guidance in the work are here gratefully remembered. Thanks are due to Professor Emil Witschi, Professor P. L. Risley, Dr. F. A. Beach, and Mr. C. M. Bogert for their kind criticism of the manuscript and to Mr. C. B. Perkins for supplying the geckos.

[2] Aided by grants to Dr. G. K. Noble from the Committee for Research in Problems of Sex, National Research Council, and from the Josiah Macy, Jr., Foundation.

[3] The personnel of Works Progress Administration, Official Project No. 265-1-97-16 WP 10, aided in animal maintenance and preparation of materials.

Altogether, almost 100 geckos were studied, but the cages usually contained the following numbers of animals: Cage 1, 4–6 females; Cages II, III, IV, 4 males each. Individuals were identified by enamel-paint spots and cutting of toes. An additional large cage with floor space of 32×67 inches served for a study of aggregation tendency, from October 28 to December 12, 1939.

EXTERNAL FEATURES

Detailed descriptions of the external characters of this species have been given by Cope (1900) and Van Denburgh (1922). The following summary is supplemented by our own observations and those of Noble (1921), Noble and Bradley (1933), Wellborn (1933), and Klauber (1939).

Van Denburgh (1922) describes the color pattern of a living specimen of *C. variegatus* as follows: "Across the back, five wide bands of dark walnut brown, palest centrally and separated from one another by dull Naples yellow bands of half their width. The tail is similarly cross-banded. . . . ." The pattern is variable; many specimens are spotted rather than banded, but the differences are not related to sex or age.

Cope found the body lengths of the adults to be 57–68 mm., with a thick tail almost as long (Fig. 1). Males are somewhat smaller than females and may be distinguished by a pair of short spines on each side of the base of the tail, curved a little upward and forward like spurs. A second pair of ventral bones lie free under the subcloacal epidermis in close association with the transverse openings of two peculiar pouches, one over each hemipenis—the subcaudal sacs (Fig. 2). The latter are lined with thin, loose, mechanically eversible squamous epithelium and not filled with erectile tissue as Cope supposed. Males also exhibit (Cope) "a series of well-defined preanal pores in the centers of six or seven large circular scales." These are the openings of glands which secrete small wax cones. The females possess all the above structures in various degrees of development. Largest are the subcaudal sacs which are quite similar to those of males (Fig. 3). Although the special preanal scales are present, they have no pores and do not secrete the wax cones. The spurs are reduced to small epidermal elevations.

Various interpretations of the functions of these structures have been offered on the basis of their appearance in preserved specimens. Wellborn (1933) assumed that the bones lying at the openings to the sacs are protective and called them the *ossa apoclistica*. She ruled out a copulatory use of the spurs, since they are directed sharply upward and outward. Noble and Bradley (1933) suggested that the subcaudal sacs of the male might function to quiet the female during copulation. They observed in *Tarentola* that the curved bones at the openings to the sacs apparently aid them to gape widely. However, Wellborn considers that the sacs "vielleicht dienen zur Aufspeicherung des Samens oder irgendwelcher Sekrete." The present study of the copulatory pattern of *C. variegatus* has resulted in an entirely new conception of the functions of these specializations.

COURTSHIP AND MATING

Under our laboratory conditions the banded gecko was sexually very reactive during the summer period of observation. Thirty copulations were recorded; the ratio of these to the total of observed courtships was approximately 1 to 12. The complete initial performances leading to 15 of the matings were fully ob-

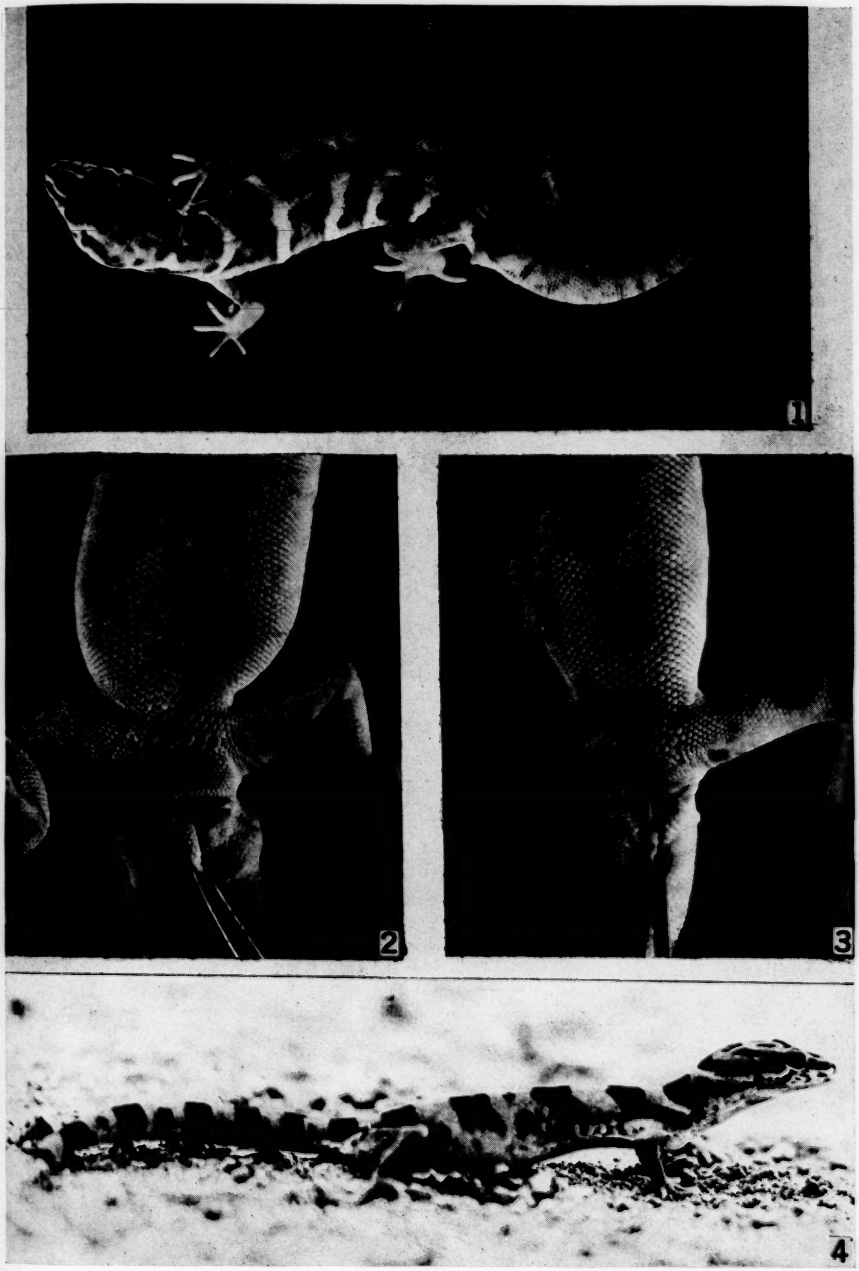
PLATE I

FIG. 1.—External features of the male gecko. Note markings and thick tail with spurs at base.

FIGS. 2 and 3.—Epidermal specializations at level of cloaca of male and female. Note the six enlarged preanal scales, openings of two postanal sacs over hemipenal swellings, and two dorsally projecting spurs. In the female (Fig. 3) the enlarged scales are not perforated by cones, spurs are represented by small epidermal elevations, but sacs are well formed.

FIG. 4.—Side view of a walking female, showing characteristic manner of holding head up, as contrasted with head-down, investigatory walk of the male (Fig. 1).

PLATE I



served, and a composite pattern may be described from these and also from the many unsuccessful courtships. The male begins an investigation of a female with nose to ground, body low, and tail waving (Fig. 1). He may poke with his snout or lick with tongue and then bite or immediately pounce from a short distance and bite at tail, leg, flank, shoulder, or neck (Fig. 5). Seizing hold, he pushes jerkily forward in a tempo which resembles the strut of the male *Anolis*. If the female does not struggle too much, the male lets go and moves up toward the neck, often biting at flank or shoulder on the way and repeating the jerky movements. As indicated by Table 1, the first grip is most frequently at the tail, while the final mating grip is at the neck. The basic elements of this pattern are (1) a preliminary grip, (2) jerky courtship movements, and (3) the mount and shift of grip to the neck region. Courtship differs from the fight pattern in that the latter involves tense body posture. The bite in fighting is not an attempt to hold on and is not followed by jerky movements typical of courtship.

TABLE 1

POSITION OF MALE'S GRIP ON THE FEMALE AT INITIAL COURTSHIP AND AT MATING

Grip	Tail	Flank	Shoulder	Neck
Initial	8	1	1	5
Final	0	0	3	12

The initial procedure is termed "courtship" because it is that part of the mating behavior which depends most directly upon the female's receptivity. A significant difference between the courtship of *Coleonyx* and that of diurnal species like *Anolis* is the dependence of the whole pattern in the former upon direct contact. *Anolis* males display at a distance and approach the female with a strut which consists of rapid bobbing movements of head and shoulders, continued as the male moves forward. This type of approach gives the female ample opportunity to escape *before* being gripped and anoestrous females are able to evade the male with ease. In the gecko, contact is the first essential element, after which the male performs strutlike movements. The female signifies willingness to mate by a minimum of resistance. She is as large as the male and easily escapes if anoestrous. Often she will reach back and bite her suitor, and the pair will whirl completely around before the male lets go. The female has never been observed to respond to courtship with the fighting pattern, but very occasionally she may rise high in a posture very like that used in fighting and maintain it only momentarily as an avoidance reaction. The courting male then may pass directly under her without touching.

Although no definite response indicative of oestrus has been noted, such as the neck-bend of female *Anolis* (Noble and Greenberg, 1941), there is other evidence of the necessity of co-operation. Anesthetized females cannot be mated with, although completely passive, since the male is unable to get his tail properly placed underneath. Such a baffled male has been observed to continue his courtship for long periods of time, even reversing position so that at times he gripped the tail region of the female and curled his tail around her neck. We repeated this experiment 20 times with the same result, namely, that the male cannot copulate with a female which does not respond. This critical response can be duplicated by simply lifting the prostrate female's tail and holding it up and against the male while he completes his pattern.

PLATE II

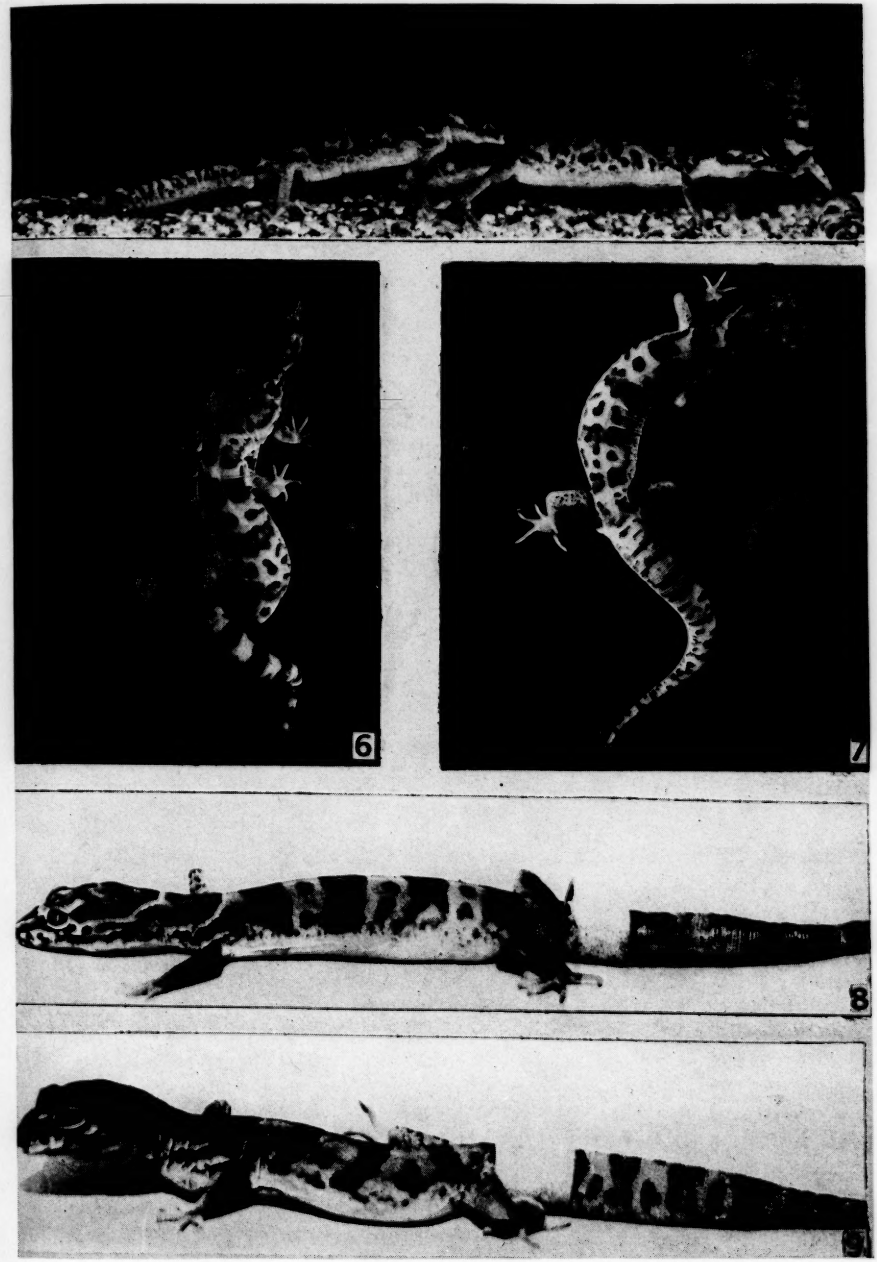
FIG. 5.—Initial courtship grip of the male. Female at extreme right is being gripped at shoulder by center male, who in turn is gripped by a second male. Note swollen cloacal region of the latter.

FIG. 6.—Dorsal view of copulation position assumed by a mating pair. The male maintains a grip on neck skin of female and a second grip on tail-base with left hind leg. The foot of this leg rests upon her thigh, while the left foreleg presses against her side.

FIG. 7.—This same male just after mating, showing the left hemipenis still everted. Note relationship between left spur and hemipenis.

FIGS. 8 AND 9.—Male and female with tails broken off and exchanged for the purpose of testing reactions of active males to them.

PLATE II



After a male *Coleonyx* has begun the courtship performance, little short of physical injury appears capable of interfering with him. It was found possible to get within very close range of a mating pair, throw spotlights upon it, remove obstructions, interpose objects such as a pencil, and even to pick up the pair and transpose them to the stage of a binocular dissection microscope without interrupting the mating. Often a particular position could be verified by turning them over. From direct observations at very close range, the copulatory pattern can be outlined as follows: Having mounted and taken a grip on neck or shoulder, the male begins to search with his hind legs for the mating position (Fig. 6). At the same time, the male's swollen cloacal region rubs transversely across the female's tail. The swelling consists of a portion in front and one back of the cloacal opening; the preanal part bears the enlarged scales and projecting comb, while behind are the subanal sheathed hemipeni. In response to the male's rubbing and palpating movements, the female raises her tail base. Thereupon the male brings his tail underneath, and this causes her tail to swing to the ipse-lateral side, pressing both close together. Then the male draws his *spur* or *hook* of the same side (Fig. 7) convulsively across her cloaca a number of times, in an attempt to secure a hold in the loose skin below it. Sometimes the hook lodges within the female's subcaudal sac but most often between and slightly below the two sacs. The pull of the hook draws the female's lower cloacal lip back and with a final thrust and pressing together by the male, the hemipenis everts, touches the lower cloacal lip, and causes it to reflex farther back, thus opening the cloaca to allow intromission.

Only one hemipenis enters the cloaca; the other remains swollen and sheathed, although there must be considerable pressure upon it. The male's subcaudal sacs now come into use. During the process of rubbing hook across cloaca, the sacs do not touch at all. Hence, traction upon the loose skin of the sac or any quieting effect due to suction are both entirely ruled out. When the hook has imbedded itself, the opening of the sac on the same side is completely obliterated by folding, but the one on the off side everts and becomes swollen. Therefore, it appears that the eversion of this sac acts to relieve pressure on the inactive hemipenis. It is also possible that the other sac provides freedom of rotation for the expanded base of the spur when the latter is swung back by the male's final copulatory thrust.

During the mating both are quiet and may be picked up and handled. If the female moves, the male performs a series of jerky and twisting movements of the head while maintaining the neck grip. These resemble the initial courtship movements and appear to quiet the female and prolong the mating.

Each of the male's copulatory reflexes evokes a reflex response on the part of the female. Rubbing the preanal comb and swollen cloacal region across her tail causes it to lift up. As his tail goes under, hers swings toward the ipse-lateral side. When the expanding hemipenis touches her lower cloacal lip, it reflexes back, and the cloaca gapes widely. That these responses of the female are actually reflex is easily demonstrated. Each can be elicited by mechanical manipulation. If a female is held loosely by the neck skin and the tail-base is gently stroked, the tail invariably lifts and can be caused to swing to left or right by touching the skin on the one side or the other. Similarly, touching the lower cloacal lip with

finger or instrument elicits the gaping reaction. These responses were secured in oestrous females and some apparently not so receptive, but no attempt was made to correlate the ease of their elicitation with sexual receptivity.

It may be noted that the accessory structures are brought into play at definite places in the pattern. For example, it was observed that the preanal wax cones touch the female only during the transverse rubbing movements which elicit the elevation of the female's tail. When underneath her, the male's hind region is so twisted that only the dorsally projecting spur actually touches, and copulation follows immediately upon its successful anchoring. To test the function of the cones, anesthetized females were placed in the cages, and, upon securing mating attempts by active males, the observer placed his finger under the male in place of the female. The male was not interrupted and continued the palpating, rubbing movements which the observer was able to feel. It appears that the cones rub across the female's tail, and this seems to be their sole function. However, his swollen cloacal region also makes contact, and it is possible that this alone is sufficient for the purpose and the cones probably are accessory.

In order for copulation to succeed, the spur must take hold not far below the cloacal opening. If collodion is placed on that area, making it hard and smooth, the male is completely unable to mate. In one exception, however, the male mated with an anesthetized male in a most unusual manner. The hook imbedded itself in the angle of the subject's hind leg, and the courting male performed his thrust; the hemipenis expanded and came to rest on the former's undersurface without contact with his cloaca. Ejaculation without any observable movements followed while the pair was being examined. Apparently contact with the cloaca is not essential for ejaculation.

The presence of the spur and its action during the 'mating seems to have made possible a new mechanism of initiating expansion of the hemipenis. The male's final thrust rotates the spur, and its enlarged base, which lies under the skin over the hemipenal swelling, exerts pressure on the hemipenis, which then expands. Pressure is also exerted by the tight leg grip but this does not bear as directly on the hemipenis as that brought to bear by the spur.

SEX RECOGNITION

An interesting problem related to the courtship performance is the question of how a courting male distinguishes the female as a mating object. The term "sex recognition" has generally been employed by naturalists to denote this phenomenon. At some point of an encounter with another male or a female, the male lizard reacts to definite cues which release, in Lorenz' sense, either of his two main patterns of social behavior.

Lorenz (1935) postulates a lizard level of sex recognition on the basis of the work of Noble and Bradley (1933). At this level it is supposed that a male is recognized as such because he responds to challenge with fight, while a female does not fight and therefore is courted. Although response generally plays an important part, later studies have indicated that, at least in *Anolis*, it is far from the sole factor (Noble and Greenberg, 1939). In *Anolis* such physical differences between the sexes as the size and appearance of the adult lizards are sufficient to release the male's corresponding pattern, while behavior differences are not essential, although they are effective aids.

A peculiarity of the gecko, not characteristic of *Anolis*, was early noted. Once a courtship is begun, the male *Coleonyx* quickly becomes "overactive" if the mating sequence is broken by escape or removal of the partner. No discrimination is then observed. Dominance becomes momentarily thwarted, since a subordinate male will court the dominant until the latter reasserts his position. The courting male's reactions become quick and violent. A typical case may be cited:

7/12/39—Cage 4: Anaesthetized male and female placed in cage 4. Resident male investigates the female and promptly bites her in courtship, struggles to mate. He is temporarily removed, but immediately upon return to the cage, he makes for the female and bites her again. The male is taken off and bites my finger repeatedly, making courtship movements. Put near a group of males, he dashes at and bites two of them. He comes upon the anaesthetized male and grips him.

After the initial courtship, a generalized mating attempt persists for some time. This may be considered in the light of other elements of the life-cycle, namely, nocturnal habits, necessity for close contact before courtship begins, and the place of the "strut" in the courtship pattern. Unlike *Anolis*, the display of *Coleonyx* is not performed at a distance from the female, and strutting courtship movements do not begin until a grip is taken. Overactivity or persistence of mating attempt upon interruption during courtship may be considered an adaption to secure completion of the mating when contact with the partner is accidentally broken.

During the course of the study it was observed that, when not hyperexcited, male *Coleonyx* readily distinguish other males from females. A newcomer male, when approached and investigated by another male shows fight, whereas a female does not. All males which fight are thereby "recognized" or responded to with the fight pattern. Another difference is that the female, when moving past a male, has her head up (Fig. 4), while the male advances with head down and tail curling. The oestrous female does not run away at approach, and this receptivity and neutral walk might excite the male to courtship. The sex of the newcomer male, however, might be indicated by his investigative walk and fight pattern.

In order to test the extent to which behavior is essential to sex recognition, anesthetized subjects were introduced into the cages. Males and females treated with ether were used, either singly or paired. In 7 out of 12 cases prostrate males were investigated and courted; the other 5 trials resulted in indifference. It appears, therefore, that motionless males are not fought with but instead are most often courted. Eight nonresident females were etherized and placed in the males' cages. Seven of these elicited immediate courtship and prolonged mating attempts, but the remaining case was oddly different. The female had been placed on the sand with head lowered. One of the males encountered her from the fore and immediately bolted. A second male approached but did not court, and the dominant male of this cage, coming upon her from the rear, nudged her tail but did not court. Possibly the odd posture may have been significant.

Six anesthetized pairs (male and female) were placed at various times in the cages, about 6 inches apart and parallel to each other. A total of 12 reactions to these pairs by introduced males were recorded, and in 9 the female alone was bitten and courted while the male was ignored. In 4 of the latter 9 contacts the active male had first thoroughly investigated both, returning to court the female.

These observations suggested that the female might be identifiable as a mating object through touch or smell or other chemical stimulus whereby she differs from the male. To test this possibility, a further modification of the experiment involving discrimination between pairs was developed. Four different male and female pairs were anesthetized, and their tails were broken off near the base and interchanged; the female's tail was attached to the male and vice versa. Since a majority of the courtship approaches are from the rear and begin with a grip on the tail, it was thought possible to discover whether the female's tail alone, when borne by a male, would be attractive. The two were placed in the cage while still motionless but usually revived after a time and moved sluggishly around dragging the foreign appendage after them (Figs. 8 and 9). Although the anaesthetized pairs were investigated a number of times while prostrate, only 3 actual mating attempts were observed, in all of which the active male gripped the female tail attached to the male and courted with it in his mouth. Twelve positive reactions to the test animals were observed while they were moving around the cages. In 5 cases the female was gripped on some portion of the body above the false tail, while the tail itself was gripped only once. Significantly, the male was courted 5 times, and each time the "female" tail was gripped.

From these few cases it would appear that male *Coleonyx* respond to some chemical stimulus emanating from the skin of the female, which acts to reinforce such negative behavioral cues as absence of fight, neutral walk, and receptivity to courtship.

FIGHT AND DOMINANCE

The males of Old World species of geckos are known to be vicious fighters, in constant dispute over territories during the breeding season. Fischer (1887) described such home defense by male *Hemidactylus* and *Gymnodactylus* against newcomers placed within their terraria. Geckos have been reported to emit various cries while fighting and even during courtship (Loveridge, 1923). However, there is no clear evidence of a relation between these sounds and either behavior pattern.

We have 46 separate records which could be interpreted as fights. These were taken during 18 observation days between June 2 and August 10. During this time the subjects were 9–16 males and almost as many females. The small number of fights observed indicates that this particular gecko is much less belligerent than iguanids and probably less than the Old World geckos. A single male *Anolis* may fight that often in several days, when given sufficient contact with male rivals.

In the fight pattern of *Coleonyx*, the male rises high on all four legs and arches his back, with head held low and throat swelled. If the opponent does not immediately run or move away, the attacker stalks around, nudging at neck, flank, or tail. Both males may do this, and occasionally they are observed to stand side by side and touch each other, first with flank and then shoulders. The fights are of very short duration. Often an aroused male stands with head up and throat swelled, then dashes directly at his rival and bites him; he makes short rushes and stops and then rushes and bites again. Voice does not enter directly into the fight pattern but is only heard

when one of the geckos is bitten. They will also squeak when handled by the observer. Thus *Coleonyx* rarely emits sounds and differs markedly in this respect from the Indian geckos, whose calls come so regularly that the natives wager upon their frequency during specified intervals (Barbour, 1926).

Males may rest close together without antagonism. Homosexual courtship is a frequent cause of fighting. The courted male retaliates with the fighting pattern, especially since courtship involves biting to secure a hold. Although fights are relatively brief and mild, dominance of one male over a group of males is the rule. In Cage II, one male won 25 out of 27 total combats. In another group 11 fights were recorded, all of which were dominated by a single male. The same situation was noted in the remaining cage containing males, although records of the fights were not kept. Since one male did all of the fighting, "pecking order," or relative dominance, could not be determined.

The females were never observed to fight. It is interesting that in this species where sex dimorphism is not very pronounced and the female may be even slightly larger than the male, the former does not fight during the breeding season. Dominance of male over female is essential to mating among lizards; and therefore, without a size superiority of the male, the aggressiveness of the female would be an interfering factor.

This type of dominance suggests the possibility of territory in the field. However, the conditions under which territories might be maintained by *Coleonyx* are very different from those of diurnal, arboreal lizards like *Anolis* or of fence lizards (*Sceloporus*). Unlike *Anolis*, there is no threat at a distance (dewlap), and fights occur only at very short range, depending upon contact. Since these geckos are not very pugnacious, territorial spacing of the males would seem to depend mainly upon size of population of a given area.

AGGREGATION

The survival of a nocturnal species is furthered by any mechanism which brings individuals together. Hence it was of interest to note a strong tendency of the geckos to form groups under shelters during the lighted period of their experimentally arranged day. This attraction was not merely sexual, since males were often found near other males as well as females.

To investigate this social factor under laboratory conditions, 25 stock animals (16 males, 9 females) were placed in a large cage with floor space of 32×67 inches. A variety of shelter was distributed over the sand floor of the cage: four large pieces of moss, five strips of hollow bark, several broken flower pots and assorted pieces, two large water dishes, and two smaller food dishes. The material was arranged symmetrically, with approximately equal positional value for similar pieces (Fig. 10). The geckos were maintained within a greenhouse under ordinary lighting and a constant temperature of about 28° C. Records were taken at 9:00 A.M., when none of the animals were on the surface. Eight such counts were made between October 28 and November 10, and another series of 7 from November 14 to December 12. Each piece of moss or bark was raised in turn, and all the geckos under it were identified. The animals reacted slowly and were easily secured without confusion. After the first eight counts, identification marks (paint spots)

began to be lost as the result of shedding, and therefore toes were cut for a second trial.

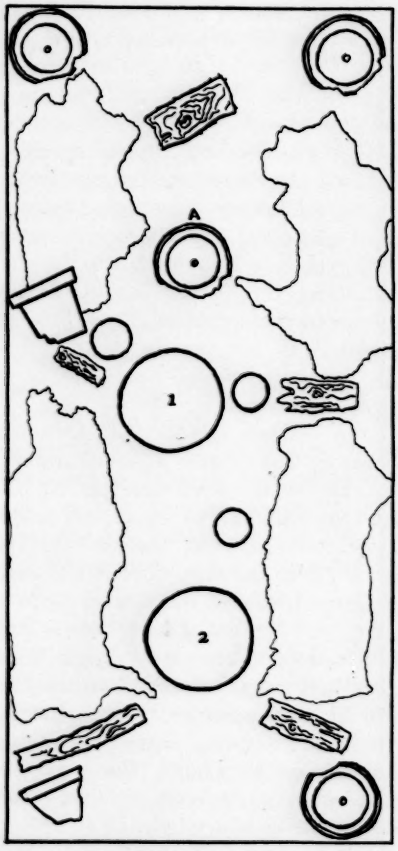


FIG. 10.—Scale drawing (14:1 inch) of the floor of the cage used in studying aggregation tendency. The cage had a floor space of 67×32 inches and contained 16 males and 9 females. Four large pieces of green moss, a number of up-ended flower pots with broken margins and several halves, five pieces of bark, two water dishes, and three small food dishes were symmetrically arranged to give equal positional value to similar pieces.

Table 2 presents the sizes of groups found during these fifteen counts. Almost invariably the majority of the animals were bunched together in several large groups. When the moss or bark was lifted, they were observed to be close together, not merely adjacent to one another. The evidence is clear that under the conditions of the experiment a strong aggregation tendency was manifested by the lizards. Only 1 animal was found alone more than once in succession. Our data applied equally to both sexes.

TABLE 2

AGGREGATION TENDENCY, AS MEASURED BY SIZES OF GROUPS UNDER VARIOUS DAYTIME SHELTERS

TRIAL 1

Oct. 28	Oct. 30	Oct. 31	Nov. 1	Nov. 2	Nov. 3	Nov. 7	Nov. 10
7	5	9	5	7	9	9	7
7	5	4	4	5	4	4	6
6	5	2	3	3	3	3	3
2	4	2	3	2	3	3	3
1	3	2	3	2	2	2	2
1	3	2	3	1	2	2	2
1		1	2	1	1	1	1
		1	1	1			
		1		1			
				1			

TRIAL 2

Nov. 14	Nov. 20	Nov. 24	Nov. 28	Dec. 5	Dec. 8	Dec. 12
8	11	6	8	7	8	8
4	4	6	5	6	8	6
3	2	4	4	5	3	3
3	2	2	3	3	1	2
2	2	2	2	1	1	1
1	1	1	1		1	1
1	1	1				1
1	1	1				
1						

Certain areas of the cage served as shelter for the great majority of the animals, while others of similar appearance did not have any groups. For example, under the large strip of moss at upper right of Figure 10 there often were two distinct groups, whereas the other three similar pieces of moss held few or none. Water dishes 1 and 2 seemed identical,

but No. 1 never concealed any animals, while No. 2, when lifted, usually revealed a group. Of the two pieces of bark at the lower portion of the cage, the smaller right-hand piece almost always was the shelter for several or a group of geckos, but not so the similar and larger left-hand piece. The broken up-ended flowerpots were much-used locations, but even these differed in attractiveness. The center one at *A* was often used, while the others were seldom occupied by more than a single animal. This use of one site in place of others suggests persistent residence within a particular area. It may be assumed that a nucleus of each group was constant to one location and attracted the wanderers who were thus prevented from starting new groups at unfamiliar shelters.

In general, two opposing tendencies may be noted. *Dispersal* is furthered by courtship and dominance, since the males drive out other males and anoestrous females. However, *aggregation* as seen at daytime shelters is a factor even during the breeding season. Thus the gecko differs from strongly territorial species like *Anolis*, where a single male and a small group of females occupy an area during the breeding season but at other times isolation of individuals is the rule, except in crowded regions. *Anolis* are generally isolated, coming together for breeding periods, during which the males are widely spread over territories; but nocturnal *Coleonyx* are probably more gregarious when not breeding than during the breeding season, when dispersal factors come into action.

SUMMARY

1. The mating pattern of the male gecko is a timed sequence of events in which each of a number of epidermal specializations has a definite place. The male's copulatory reflexes elicit corresponding reflex reactions of the female, whose co-operation is essential for copulation.

2. "Sex recognition" in this nocturnal species depends primarily upon behavioral cues. Anesthetized males are treated as females, but experiments concerned with discrimination between a male or female member of an introduced pair suggest that other stimuli may be present which involve the chemical senses.

3. Absence of sex dimorphism of size and display characters has not favored the development in the female of fight, dominance, or territory. Thus *Coleonyx* differs from diurnal lizards, such as *Anolis*, in which dimorphism is present and is accompanied by fighting among the females resident within a male's territory.

4. Two opposing forces are seen to regulate the social system of the banded gecko. *Dispersal* during the breeding season is furthered by fighting among the males, which leads to dominance and perhaps territory and by courtship of anoestrous females. *Aggregation* as manifested under laboratory conditions is a strong factor even during the breeding season, when it probably aids reproduction, since the male's initial courtship performance is at close contact rather than at a distance.

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NONSYMBIOTIC UTILIZATION OF CARBOHYDRATES BY THE TERMITE, ZOOTERMOPSIS ANGUSTICOLLIS

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In a recent paper Hungate (1939) has outlined a new theory concerning the mechanism of the symbiosis between the termite and its intestinal protozoa. His analyses indicate that the microörganisms degrade cellulose to glucose and then dissimilate the latter anaerobically to hydrogen, acetic acid, and other, unidentified products. The acetic acid and perhaps other substances are then absorbed and oxidized by the termite. If this scheme represents the normal course of events—and Hungate's evidence is strong—then it becomes pertinent to inquire whether or not the termite can utilize ordinary sugars and polysaccharides and, if so, to what extent.

It is possible to estimate carbohydrate utilization by direct feeding to both normal and defaunated termites. The method employed is that described by Cook and Scott (1933). A stiff agar gel (5 per cent) is used as a substrate into which is incorporated the food substance. The animals, *Zootermopsis angusticollis* (Hagen), excavate the solid agar and ingest it very readily. Given a properly balanced diet, including vitamin extracts, they will live indefinitely.

After exposure for several days to a diet of agar plus an experimental substance, the gas exchange of the termites is measured manometrically at 24° C. in a Warburg apparatus of standard design. This includes oxygen consumption, carbon dioxide, and hydrogen production.

The evolution of hydrogen affords a convenient index to the activity of the protozoa themselves, since, according to Hungate, all carbohydrate metabolism is accompanied by the formation of this gas. For the termite itself we may employ the Respiratory Quotient.

Numerous investigations have made it clear that the normal R.Q. of the termite, when subsisting on cellulose, is between 0.9 and 1.0. In the post-absorptive state or during mild starvation the R.Q. sinks to the region of 0.70–0.75. This is in conformity with general experience, for under these conditions both warm- and cold-blooded animals tend to draw directly or indirectly upon their reserve of fat.[1] If, now, the animals are placed upon a substrate of agar plus a carbohydrate and the latter is metabolized, then the R.Q. should approach unity. If, on the other hand, the substance is inert, the R.Q. should remain at a low level.

The possible participation of the microfauna in making carbohydrate available to the host may be determined by two methods.

1. If in the normal termite the hydrogen production disappears or sinks very low, this is prima facie evidence that the protozoa are not active and may be absent. Consequently, they cannot be a factor in metabolism. Conversely, if the

[1] In order to check the presence of fat in the termite several analyses were made through the courtesy of Dr. Cecil Entenman of the Division of Physiology. Twenty-two determinations, each made on a lot of 2–3 gm. of termites, gave a mean total fatty acid content of 6.4 per cent. The fat store, therefore, appears adequate for subsistence over a considerable period.

hydrogen production is high, the presumption is that the protozoa are actively attacking the ingested material.

2. Termites may be defaunated. When they subsequently show a high R.Q., it is evident that the transformation of the carbohydrate is due to the termite alone and not to any symbiotic participation by other organisms. When they still show a low R.Q., it is similarly clear that they are unable alone to utilize the foodstuff.[2]

Starvation.—As an initial experiment, two groups of termites were placed on agar without supplement. Group 1 was normal. Group 2 was defaunated by oxygen at 30 pounds pressure applied for 4 hours. After 7 days, Group 1 showed no hydrogen production and an R.Q. of 0.721 (see Table 1). Group 2 showed an R.Q. of 0.735. Microscopic examination yielded no protozoa in any of the groups. It is apparent that in the absence of any external food supply the termites were metabolizing endogenous fat and were slowly starving.

Cellulose.—Cellulose was incorporated in agar as finely divided filter paper. With normal termites a series of four tests showed a mean hydrogen production of 7.0 cu. mm. per gram termites per hour at 24° C. This value is below that usually found with termites on wood but is still sufficient to demonstrate the existence of an active protozoan fauna. At the same time the mean R.Q. was 0.995. This result was, of course, to be expected, since it represents an approximately normal metabolic condition. A parallel group of defaunated insects yielded an R.Q. of 0.757 with no measurable hydrogen production. This likewise is reasonable, since in the absence of protozoa the termite is unable to degrade cellulose.

Glucose.—Three groups of normal termites were placed upon agar containing 8 per cent glucose. Group 1 showed only minute traces of hydrogen at 20° C., but Group 2 produced the gas at the rate of approximately 9.0 cu. mm. per gram termites per hour at 32° C. Group 3 yielded approximately 1.9 cu. mm. per gram per hour at 24° C. The protozoa were, therefore, active, but at an abnormally low rate. The mean respiratory quotients of the termites for the three groups were, respectively, 0.976, 0.902, and 0.967. Hence the hosts were utilizing the carbohydrate.

The suggestion might immediately be offered that, even with reduced activity, the protozoa were metabolizing the glucose to acetic acid, which in turn was being absorbed by the termite. To answer this question a third group was defaunated prior to exposure to the glucose diet. The mean of six determinations yielded an R.Q. of 0.958, almost exactly that obtained with the normal animals. It may, therefore, be stated that the termite *can* absorb and oxidize glucose directly. However, this conclusion, per se, does not exclude the possibility that ordinarily the microfauna subjects glucose (from cellulose) anaerobically to further decomposition. It must be remembered that Hungate (p. 245) maintains only that "most," not all, of the glucose undergoes this type of dissimilation. The alternative is still open that some of it is absorbed and used directly. To accept such an explanation would imply the existence of two possible modes of securing carbohydrate. The quantitative relationship between them would then depend upon the character of the diet, the state of the protozoa, the oxygen tension, and, perhaps, other factors.

[2] It is quite possible that the defaunation does not eliminate the bacteria. However, as Hungate (1939) has shown, the participation of gut bacteria, in the quantitative sense, is unimportant and for the present purposes may be neglected.

TABLE 1

DATA SHOWING OXYGEN CONSUMPTION TOGETHER WITH CARBON DIOXIDE AND HYDROGEN PRODUCTION

Each determination represents the mean for 2–5 vessels containing approximately 1 gm. of termites. The temperature was 24° C., unless otherwise specified.

Substrate	Condition	Oxygen Consumption Cu. Mm. O_2 / Gm.×Hr.	Carbon Dioxide Production Cu. Mm. CO_2 / Gm.×Hr.	Hydrogen Production Cu. Mm. H_2 / Gm.×Hr.	R.Q.
Agar	Normal	251	180.5	0.5	0.719
		203	153	2.5	0.753
		248.5	175.5	0.0	0.706
		268	192	2.0	0.716
Mean		*242.6*	*175.0*	*1.25*	*0.721*
Agar	Defaunated	279	212.5	0.0	0.761
		238	168	0.0	0.706
Mean		*258.5*	*190*	*0.0*	*0.735*
Cellulose	Normal	345.5	353	6.1	1.023
		246	242.5	7.8	0.987
		251	229	7.7	0.913
		195.5	207	6.4	1.058
Mean		*259.5*	*258*	*7.0*	*0.995*
Cellulose	Defaunated	264	199	0.0	0.754
		255	194	0.0	0.761
Mean		*259.5*	*196.5*	*0.0*	*0.757*
Starch	Normal	353	332	0.0	0.941
		288	267	0.0	0.927
		253	246.5	0.0	0.974
		187	183	0.0	0.978
Mean		*270*	*257*	*0.0*	*0.953*
Starch	Defaunated	259	215	0.0	0.829
		253	215	0.0	0.850
		280	244	0.0	0.873
		233	223	0.0	0.957
		223	177	0.0	0.793
		225	265	0.0	1.178
Mean		*245.5*	*223*	*0.0*	*0.907*
Sucrose	Normal	325	306	14.4	0.941
		312	343	4.8	1.100
		332	364	6.1	1.097
Mean		*323*	*351*	*8.4*	*1.086*
Sucrose	Defaunated	308	333	0.0	1.081
		272	261.5	0.0	0.961
		336	309	0.0	0.920
		235	306	0.0	1.300
		233	231	0.0	0.992
Mean		*273*	*288*	*0.0*	*1.055*

TABLE 1—*Continued*

Substrate	Condition	Oxygen Consumption Cu. Mm. O_2 / Gm.×Hr.	Carbon Dioxide Production Cu. Mm. CO_2 / Gm.×Hr.	Hydrogen Production Cu. Mm. H_2 / Gm.×Hr.	R.Q.
Glucose I.....	Normal 20°	308	301	0.0	0.976
Glucose II.....	Normal 32°	649	585.5	9.1	0.902
Glucose III....	Normal	275	243.5	4.2	0.885
		345	347	1.5	1.008
		301	291	0.0	0.967
Mean.....	.....	*307*	*297*	*1.9*	*0.967*
Glucose.....	Defaunated	296	301	0.0	1.017
		288	279	0.0	0.968
		345	308	0.0	0.894
		325	334	0.0	1.027
		313	279	0.0	0.892
Mean.....	.....	*313*	*300*	*0.0*	*0.958*
Maltose.....	Normal	416	387	3.5	0.930
		312	265	5.4	0.850
Mean.....	.....	*364*	*326*	*4.5*	*0.896*
Maltose.....	Defaunated	297	283	0.0	0.953
		305	334	0.0	1.092
Mean.....	.....	*301*	*308.5*	*0.0*	*1.025*
Fructose.....	Normal	312	289	4.3	0.927
		322	268	7.4	0.832
Mean.....	.....	*317*	*278.5*	*5.8*	*0.878*
Fructose.....	Defaunated	416	407	0.0	0.978
		305	321.5	0.0	1.055
Mean.....	.....	*360*	*364*	*0.0*	*1.010*
Lactose.....	Normal	372.5	358	9.5	0.962
		248	284	50.0	1.145
		232.5	196.5	6.8	0.845
		287	279	14.4	0.973
		320	304	5.7	0.950
Mean.....	.....	*292*	*285*	*17.3*	*0.976*
Lactose.....	Defaunated	234	252	0.0	1.078
		297	361	0.0	1.216
Mean.....	.....	*265.5*	*306.5*	*0.0*	*1.155*
Galactose.....	Normal	192	188	7.9	0.978
		281.5	249	2.5	0.885
		187.5	161.5	4.0	0.862
Mean.....	.....	*220*	*199*	*4.8*	*0.905*
Galactose.....	Defaunated	311	289	0.0	0.930
		255	265	0.0	1.040
Mean.....	.....	*283*	*277*	*0.0*	*0.979*

TABLE 1—*Continued*

Substrate	Condition	Oxygen Consumption Cu. Mm. O_2 / Gm.×Hr.	Carbon Dioxide Production Cu. Mm. CO_2 / Gm.×Hr.	Hydrogen Production Cu. Mm. H_2 / Gm.×Hr.	R.Q.
NaAC., pH 6..	Normal	228	166	0.0	0.728
NaAC., pH 5..	Normal	264	187	0.0	0.708
NaLac., pH 6..	Normal	256	179	0.0	0.699
NaLac., pH 5..	Normal	196	135	0.0	0.689

Other carbohydrates.—If the termite is capable of metabolizing glucose in a manner analogous to that of animals in general, may it not also be able to use other sources of carbohydrate? In order to examine this possibility termites were placed upon diets of starch and sucrose.

With starch, normal animals produced no hydrogen whatever; with sucrose, the hydrogen evolved amounted to an average of 8.4 cu. mm. per gram per hour at 24° C. Thus the protozoa, although unable to metabolize starch, can split sucrose with considerable facility.

The respiratory quotients were as follows: with starch and normal termites, 0.953, defaunated termites, 0.907; with sucrose and normal termites, 1.086, defaunated termites, 1.055. Although microfaunal assistance is lacking, therefore, the insect is able to digest starch. Sucrose, on the other hand, with or without the aid of protozoa is quantitatively inverted and oxidized. This fact argues for the existence of an appropriate enzyme in the gut of the termite.

Since the final product of both starch and sucrose digestion is glucose, these substances may be assumed to undergo actual absorption in the form of that sugar. However, sucrose on hydrolysis yields fructose as well as glucose, and an important intermediate stage of starch hydrolysis is maltose. The direct availability of fructose and maltose were consequently tested.

With maltose and normal termites, the hydrogen production averaged 4.5 cu. mm. per gram per hour and the R.Q., 0.896. With defaunated termites the hydrogen production was absent, and the R.Q. was 1.025. With fructose the corresponding values for hydrogen production were 5.8 and 0.0; for the R.Q., 0.878 and 1.010.

A third common monosaccharide is galactose, derived usually from lactose. These two sugars were consequently tested. The average values (see Table 1) show good hydrogen production with normal termites and R.Q.'s above 0.900 in all cases.

From the foregoing experiments it is reasonable to assert that both the termite and its symbiotic protozoa are independently capable of deriving energy from numerous sources of carbohydrate. Since the microörganisms possess, in addition, the power to degrade cellulose, there has evolved the mutual relationship whereby the two organisms together make use of this otherwise inaccessible material. The relationship, however, is *facultative* in so far as the termite is concerned, for, as has been shown, the latter may metabolize starch, sucrose, maltose, lactose, glucose, fructose, and galactose. A corollary to this conclusion is that the termite very probably possesses a set of hydrolytic ferments capable of acting on the first four of these substances.

Organic acids.—In the light of Hun-

gate's hypothesis, it would be of interest to show that the termite could absorb and oxidize acetic acid directly. The attempt was made to feed termites this acid incorporated with agar. For purposes of comparison, a series was placed upon agar with lactic acid. Both acids were made up in 0.2 normal solution. They were adjusted with NaOH so as to give pH 6 and pH 5. The results were entirely negative. In no case was there any hydrogen production whatever, a clear indication that the microfauna was killed off. The respiratory quotients were 0.728 for acetic acid of pH 6, 0.708 for acetic acid of pH 5, 0.699 for lactic acid of pH 6, and 0.689 for lactic acid of pH 5. The termites were thus unable to utilize either acid under the existing conditions. It should be emphasized, nevertheless, that these results are in no way definitive. The pH may have been too high or too low, for we know nothing of the actual pH in the gut or within the protozoa. The cation used to neutralize the acid may have acted as an inhibitor. The actual concentration of the acid may have failed to correspond to that normally present. A more detailed investigation with further refinements of technique will be necessary in order to establish whether or not the termite can assimilate acetic acid administered directly in the diet.

Summary.—By the use of agar diets with carbohydrate supplements, it has been determined that *Z. angusticollis* shows a typical carbohydrate R.Q. with starch, sucrose, maltose, lactose, glucose, fructose, and galactose. This is taken as evidence that these substances can be digested, absorbed, and oxidized by the animal independently of the intervention of symbiotic organisms.

The appearance of hydrogen in undefaunated termites indicates that the protozoa can metabolize the sugars tested but cannot attack starch.

Acetic and lactic acids, in the form of their sodium salts, could not be utilized by the termites.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

PATTERNS AND PROBLEMS OF DEVELOPMENT,[1] BY C. M. CHILD

Reviewed by L. H. HYMAN
American Museum of Natural History

IN THIS impressive volume Professor Child has given an extensive presentation of his life-work and general biological philosophy. Throughout his long and fruitful career Professor Child has never hesitated to attack courageously the central and most difficult problem of biology—that of organization. As the title of the book indicates, he has viewed this problem as essentially one of development. The central thesis of the book is that development is a much broader process than embryonic animal development, comprising in addition to this plant and animal budding, all types of asexual reproduction, and reconstitution and regeneration of cell aggregates, portions of embryos, and pieces of plants and animals; and any theory of development must be equally applicable to all these processes. Many of these types of development are simpler and more generalized than the embryonic development of animals and hence are of more significance for the consideration of large problems. Development is conceived as a

> series of events integrated into a definite pattern. . . . . The occurrence of these many different forms or types of development raises the question whether . . . . essential similarity of pattern is to be found in some or all of them.

As is well known, the author has made the search for such a common underlying pattern of development the object of some forty years of research.

The pattern of organization of nearly all plants and animals is an axiate or polarized pattern, and the problem of development therefore becomes a study of the characteristics of axiate pattern and the causes of its origin. The morphological and physiological characteristics of axiate pattern in embryonic development, asexual reproduction, and reconstitution are presented in chapters ii–iv. Morphologically there is seen gradation in cell size, concentration of cytoplasm, etc.; physiologically there is manifested, concomitant with axiate pattern, gradations in growth rate, rate of division, rate of respiratory metabolism, rate and type of reconstitution, susceptibility to various toxic substances, depth of staining with vital dyes, rate of reduction of oxidizing substances, differences of electric potential, etc. From this mass of data the conclusion is reached that axiate pattern in its simplest and most generalized form consists of a three-dimensional quantitative gradient in essential physiological processes, especially metabolic processes. This gradient pattern is regarded as primarily quantitative, but it is everywhere emphasized that quantitative differences must soon bring about qualitative ones. The gradient pattern is the primary organizing and integrating factor in development. The general spa-

[1] Chicago: University of Chicago Press, 1941. Pp. ix+811+224 figs. $8.00.

tial arrangement and localization of parts in development is controlled by this gradient pattern but, as is repeatedly stated, the specific kinds of organs which arise result from the specific protoplasm in which the gradient acts.

Further evidence that the gradient is a "physiologically active and effective factor initiating the order and unity of the individual" is brought forward in chapters v–vii, which concern the modification of development by experimental means in a wide range of materials. Since many different agents and conditions evoke in a variety of materials similar modifications of general body proportions and degree of regional development in relation to axiate pattern, it follows that the action of such agents and conditions can hardly be specific but must consist in relative alterations of activity of a quantitative gradient. Differential inhibition, the greater inhibition of the more active parts of the gradient, plus differential tolerance and recovery, i.e., the greater powers of adjustment of the more active levels of the gradient, serve to explain the majority of the results of experimental modification of development. Although the mass of data presented is impressive and the explanation often convincing, especially as regards the series of head forms and anterior repressions, it seems to the reviewer that the author here perhaps attempts too much.

What the reviewer has always considered as the most brilliant, convincing, and fruitful part of the gradient theory is the concept of dominance and subordination and physiological isolation and their application to asexual reproduction and regeneration. This material is presented in chapters ix and x. Distance relations to the dominant region in the budding and fission of coelenterates and flatworms are described, and the role of the dominant region in regeneration is discussed. The evidence indicates that the dominant region, as the hydranth of coelenterates or the head of flatworms and annelids, arises first and independently of other parts in fission and in regenerating pieces, and the spatial relations of the remainder of the anatomy are determined by the dominant region, which acts as an "organizer" and is itself self-differentiating.

The origin of new axiate patterns, i.e., essentially of new dominant regions, in reconstitution is treated in chapter xi, in agametic reproduction in chapter xv, and in embryonic development in chapters xiii and xv. Here are considered polarity origins in regeneration, inductions of new polarities by grafts and injuries, and regressions and inhibitions of budding and fission by alteration of dominance relations. Little definite can be said about the origin of axiate pattern in spores, spermatozoa, unicellular buds, etc. In regard to the egg, however, there is much evidence that its polarity is determined by the action of extrinsic factors during its growth. The differential action of environmental factors is the general cause of the origin of axiate patterns. The most stimulated point becomes the high point of the physiological gradient induced by external factors; this region differentiates into the dominant region with the morphology characteristic of that particular protoplasm, and other parts are localized with reference to the dominant region.

Some other theories of (embryonic) development, especially the field and organizer theories, are critically considered. The field theory is justly criticized on the ground of general vagueness. Its propo-

nents have failed to define its nature or mode of origin or action, and in many cases it appears to be only another name for the physiological gradients of the author. Much space is devoted to an evaluation of the organizer theory. The early explanation that the organizer acts by giving off a chemical or chemicals with specific differentiating action has not been supported by later researches, which have shown that a variety of tissues, both living and dead, and even inert substances may have an inducing action, that what is induced depends on the level where implanted and on the "strength" of the inductor, and that even in amphibians nervous differentiation may proceed in isolated pieces. The most satisfactory explanation of the varied results as they stand at present is that inductors or organizers have a general activating effect. The natural inductors are already parts of an organization pattern, and they apparently act by altering regions at lower levels of the gradient than themselves. The early form of the organizer theory was, in fact, only a variant of the ever recurrent "formative-stuff" idea. The reviewer is in complete agreement with the author in regarding the latter theory as wholly unsatisfactory, since chemical substances, even if they have some specific action, can act only on a pattern already present.

The book is very well written and organized and indeed a tour de force in its scholarly presentation of an immense assemblage of data. It is carefully worded everywhere in an attempt to avoid misunderstanding or misconstruction. This attempt explains a certain amount of repetition, which is also perhaps inevitable when the same or similar data are considered under different heads or in different connections. The absence of dogmatism and the fair consideration of the work of others disarm criticism. Probably the main criticism which will be advanced against the book is that it attempts to explain too much. The reviewer rather felt this in regard to the discussions of modification of development and cleavage patterns.

The book is not only a major contribution to theoretical biology but an invaluable reference work to those interested in experimental embryology, regeneration, and asexual reproduction. The great mass of data handled, the careful documentation throughout, and the extensive Bibliography cannot but make the book of wide service and value.

THE INTERACTION OF DRUGS AND CELL CATALYSTS[1]

BY FREDERICK BERNHEIM

Reviewed by KURT G. STERN
Overly Biochemical Research Foundation, New York

THIS monograph deals with the effects of certain drugs on the activity of enzymes *in vitro* and also with the action of certain enzymes on drugs. The earlier approach to pharmacological problems, which consisted essentially in establishing the site of action of drugs and in describing their effect on the function of organs and single cells, has more recently been supplemented by the use of biochemical methods and physical-chemical techniques. Thus it is

[1] Minneapolis, Minn.: Burgess Pub. Co., 1942. Pp. ii+85. $2.25.

now possible to demonstrate the more or less specific effects of certain drugs on the intricate processes of intermediary metabolism, both in the intact animal and in the isolated organ, in tissue cultures, in thin slices of tissues (Warburg manometric technique), and—last but not least—in cell-free preparations. The experimental work of the author has been chiefly concerned with the two last-mentioned lines of approach to the problem of drug action.

About one-third of the text is devoted to the action of eserine, morphine, strychnine, curare, and methylene blue on the enzyme choline esterase. This allotment is justified both by the large volume of work done on this subject and by the prominent position which choline esterase has occupied in recent years in the theories dealing with the mechanism of transmission of nerve impulses and with the nerve-muscle relationships. Another third of the book is concerned with the action of such substances as cyanide, selenium, arsenic, copper, sulfanilamide, and salicylate on a number of other enzymes. The last part deals with the attack of specific enzymes on drugs of the type of acetanilide, histamine, atropine, acetyl derivatives of morphine, adrenalin, and alcohol.

The material has been treated in a concise and comprehensive manner. The monograph is definitely useful in that it affords, perhaps for the first time, a review of our present knowledge of the interaction between drugs and enzymes. Its usefulness is further enhanced by extensive literature references.

The present status of the subject is best summarized in the author's own words:

> For most of the drugs the correlation between enzyme and pharmacological action is incomplete. For certain drugs it is better than for others, but in all cases more data are needed to explain inconsistencies and to fill gaps in our knowledge. It is, perhaps, the main purpose of this review to emphasize the difficulties in order that further work may prove or disprove the value of this particular method of approach.

THE PHOTODYNAMIC ACTION OF DYES ON THE EGGS OF THE SEA URCHIN, LYTECHINUS VARIEGATUS,[1] BY DAVID HILT TENNENT

Reviewed by C. M. CHILD
Stanford University

THE foreword by Dr. Vannevar Bush includes a brief appreciation of Dr. Tennent's work and his connection with the Tortugas Laboratory. It also calls attention to a fact, important in evaluation of the paper, that "the manuscript describing the experimental data was completed just before his death and it is to be regretted that many of his speculations concerning the interpretation of the observations have not been recorded."

The paper is a study of the photo-

[1] Washington, D.C.: Carnegie Institution of Washington, 1942. Pp. 153+40 figs.+8 pls. Paper, $1.25; cloth, $1.75.

dynamic action of some thirty-five dyes, mostly basic, several acid, on *Lytechinus* eggs, with irradiation by sunlight for short periods, usually 10 or 20 minutes, and with controls in darkness or diffuse light. In most experiments irradiation began a few minutes after, in some before, insemination. With many dyes only a few experiments with a few concentrations were performed, and these show merely occurrence and character of photodynamic action or, in case of Martius yellow and alizarin red S, its absence.

The usual extreme effect of basic dyes is "blister" cytolysis, the appearance on the egg surface of clear blisters, containing, as the author believes, cytoplasm without formed constituents. Acid dyes enter uninseminated eggs but are almost entirely excluded by the fertilization membrane. They produce vacuolation when they enter and sometimes blistering also. Thionin, though basic, acts like an acid dye. Less extreme effects are earlier or later retardation or complete inhibition of development. Great differences in toxicity of dyes were found, brilliant green, Janus green, and auramin O being extremely toxic. In general, toxicity was increased by irradiation. Cytological photodynamic effects, as determined for a considerable number of dyes, consist in lagging of chromosomes, failure of male and female nuclei to unite, alterations of mitosis, absence of asters, multipolar spindles, cytasters, etc. Data on effects of a few concentrations of glutathione, cysteine, cystine, and di-iodotyrosine included in the paper do not involve photodynamic action. The effects described are chiefly retardation of development and death at some stage in certain concentrations.

Neutral red (1:150,000) was used in more extensive experiment and analysis. The eggs were found to decrease in sensitivity from 5 minutes after insemination on. Use of various light filters showed that the effective range is in the shorter wave lengths of the visible spectrum, ultra-violet having little or no effect in the experiments performed. Increase in irradiation period, in light intensity above a threshold, and in temperature increased photodynamic effect. A neutral red solution, irradiated for 2 hours with rise in temperature during irradiation to 49° C. and change to light-yellow color, produced cytolysis in eggs subjected to it after irradiation was completed. After 10 days it was no longer effective. This experiment leads the author to conclude that a photocompound of neutral red is formed in irradiation but is unstable. However, the single experiment presented as evidence does not seem entirely adequate as a basis for the unqualified statement in the summary that photodynamic effect in general is due to formation of a photocompound.

Unfortunately, it must be noted that the paper is diffuse and loosely written. Earlier neutral red experiments in which light intensity and temperature were not controlled are as fully reported as later, more adequately controlled experiments. Also experiments with various dyes in concentrations below the effective threshold are given as much space as those with positive results. It seems to the reviewer that much or all of that material might have been omitted or mentioned in a few words as preliminary and that in general the paper would have been improved by adoption of a more concise style in presentation of data.

THE DYNAMIC STATE OF BODY CONSTITUENTS[1] BY RUDOLF SCHOENHEIMER

Reviewed by L. V. HEILBRUNN
University of Pennsylvania

THIS book of Schoenheimer's, published posthumously, is of interest to all biologists, for it develops a new concept of metabolism—a concept so important that it should doubtless influence the manner in which the physiology of metabolism is taught to beginning students of biology. For years now we have been in the habit of comparing the living system to an internal-combustion engine. The engine itself needed repair occasionally, we thought; but in the main the chemical processes going on in it were primarily those involved in the combustion of fuel or food. Actually, the engine itself is not even relatively inert. The protoplasm of the body is in a constant state of rapid flux.

The book consists of three lectures, originally delivered at the Harvard University Medical School in October, 1941, plus a bibliography of about a hundred papers. All three of the lectures are concerned with the experiments of Schoenheimer and his collaborators—experiments in which heavy hydrogen (H^2) and heavy nitrogen (N^{15}) were fed to mice and rats. These isotopes of hydrogen and nitrogen are harder to detect than are radioactive isotopes; but they have the advantage of stability, and they can therefore be used for experiments of long duration. When mice were fed fats containing heavy hydrogen, analyses of the fats of the animal indicated that within four days 44 per cent of the total fats of the mice had been replaced. This value is for all the fats of the animal—reserve or depot fats as well as the fats present in vital organs. Within an organ such as the liver, replacement is even more rapid; and Schoenheimer estimates that within a single day about half of the fats present in the liver undergo replacement.

Similarly, when rats were fed amino acids containing heavy nitrogen, the isotope speedily found its way into the proteins present in vital organs. When leucine was fed, less than half was excreted; also when glycine was fed, over half remained in the body.

Not all the proteins of the body are replaced at an equal rate. The most rapid replacement is in the proteins of the blood serum, the intestinal wall, kidney, spleen, liver, etc. In muscle proteins, replacement is somewhat slower, and it is still slower in the skin. But even in tissues as inert as tendons, replacement occurs at an appreciable rate.

One of the most interesting facts brought out by Schoenheimer is the diversity of the chemical reactions going on in the body at all times. If a mouse is fed palmitic acid containing heavy hydrogen, the isotope appears not only in the palmitic acid group but in a wide variety of other types of molecules. Molecules of fatty acids are continually being degraded, elongated, hydrogenated, and dehydrogenated. Similarly, from experiments with amino acids, it can be demonstrated that nitrogen atoms are rapidly being shifted from one amino acid to another. The protoplasm is thus always in a state of dynamic flux; a myriad of reactions occurs within it.

Schoenheimer's book is well printed and is easy to read. It can be recommended to all physiologically minded biologists, and it will probably also be useful as collateral reading for students in courses in general physiology.

[1] Cambridge: Harvard University Press, 1942. Pp. x+78. $1.75.

ON GROWTH AND FORM,[1] BY SIR D'ARCY WENTWORTH THOMPSON

Reviewed by J. W. BUCHANAN
Northwestern University

THIS revised and enlarged second edition of Professor Thompson's classic, first written in 1916, is an imposing extension of his earlier attempt to formulate a geometry of Growth and Form. Beautifully written in a prose at times approaching the poetic, rich in classical and scholarly knowledge, steeped in philosophy, the volume is one that should be read by everyone who would enjoy Science expressed as Literature. But the reading will not be easy, for interlarded with the factual is the philosophical; profound meanings are mined from sources that of themselves appear to be unproductive or insignificant. A vast store of literature has here been assembled and assimilated; the information it contains will widen anyone's knowledge of Nature.

Chapter i contains, to begin with, a dissertation on Final Causes, a treatment that warns in advance of the scope and approach of the effort to follow. In brief, after discussion of the insuperable difficulties of determining Final Cause but acknowledging that mechanism and teleology are inseparably interwoven, Professor Thompson elects to devote himself to Efficient Causes:

> My sole purpose is to correlate with mathematical statement and physical law certain of the simpler outward phenomena of organic growth and structure c. form, while all the while regarding the fabric of the organism, *ex hypothesi*, as material and mechanical configuration.

Chapter ii deals with magnitude and includes a lucid exposition of the extrinsic factors determining the upper limits of size of organisms. But the treatment of the factors that determine the minimum size of living objects is much less satisfactory; at least the reviewer is less satisfied with the author's command of his materials.

Nearly two hundred pages are required in chapter iii to develop the subject of rate of growth. The increase in magnitude of both individuals and populations is dealt with statistically; one finds here a review of the conventional growth equations elaborated and illustrated by data from many sources. Growth has been demonstrated to be a reversible reaction, at least among a number of more simple forms, and under such conditions of inanition the body form may be markedly altered. The author makes no reference to these facts, nor does he attempt to show that the conventional mathematical treatments apply with equal validity to both positive and negative growth. In chapter iv the internal form and structure of the cell furnish the opportunity for interjecting the role in form determination of surface forces, but Professor Thompson's discussion goes much further than that. Included in the chapter is a fairly comprehensive unification of earlier and present views concerning the nature of spindle formation, chromosome form, mitosis, and cell division. The author establishes certain formulas applicable to the forms of cells and calls in support descriptions of form of a great variety of cells.

The factors underlying boundary states and the formation of membranes furnish the starting-point for a dissertation on tissue formation in chapters v–viii. Here we are led into an extensive treatise on Solid Geometry with particular reference to the roles played by external pressures in determining cellular

[1] Rev. ed., illustrated. Cambridge: Cambridge University Press; New York: Macmillan Co., 1942. Pp. 1116. $12.50.

form in tissues. This geometry is extended to include developmental patterns in gastrulae and in cell aggregates; even the veination of the insect wing, the combs of bees and wasps, and the development of the pollen grain are incorporated into the general picture. Since four extensive chapters are necessary to develop this theme, obviously a brief review cannot do justice to the large mass of material with which the author deals or to his meticulous care in fitting together into a common geometry the diversity of cell types to which he alludes.

The forms assumed by concretions, e.g., ear ossicles, spicules, and spicular skeletons, furnish the material for chapters ix and x. Here the author seems to encounter greater difficulties in unifying his material than in immediately preceding chapters. He acknowledges his predicament in the following words:

> But every now and then we come to deep-seated signs of protoplasmic symmetry or polarization which *seem* to lie beyond the reach of ordinary physical forces. It by no means follows that the forces in question are not essentially physical forces . . . . [p. 732].

It appears to the reviewer that throughout these and other chapters the author labors mightily at times because he lacks a physiological concept of polarity.

Chapters xi and xii are elaborate mathematical treatments of spirals, into which are brought the shells of mollusks, of Foraminifera, of opercula and organic spirals generally. At this point one is tempted to recall that the Greeks sculptured a regular spiral in the Corinthian capitals but did not possess the mathematical tools to express such a spiral, and one wonders if the author has lost sight of the possibility that organic spirals merely represent measurable relations. Causality here seems remote, although the materials afford an ample fund for the exercises of the mathematician. The subject matter of chapter xiii is somewhat different, for in this section the forms of horns, teeth, beaks, and claws engage attention. The chapter is comparatively brief but, nevertheless, includes analyses of patterns assumed by a considerable variety of structures. Chapter xiv is also brief and is devoted to leaf arrangement as a function of mathematics. In chapter xv the forms of eggs and other hollow structures, such as blood vessels, are analyzed.

In chapter xvi the author returns from the particular to the general and in an impressive account develops the thesis of mechanical efficiency and the conditions of form and structure in the organism. Here we are given a lucid, although general, account of skeletal structure in relation to stresses, strain, and elasticity. "Adaptation" is rightly regarded as a cliché that too frequently is resorted to, to effect the referring of difficult problems to a vitalistic explanation. Professor Thompson avoids this by electing to speak of "mechanical fitness."

The final section, chapter xvii, seems to the reviewer to contain the essence of the long and more or less leisurely thesis and to derive the most significant generalization of the whole volume. The chapter is devoted to comparison of related forms, largely by the method of coordinates. Fundamental differences in these forms are thus revealed, and the author has his answer to the problem posed by the Darwinian theory, namely, that there can be no intergrades, that missing links are in reality not missing; the observed gaps actually represent wide or discontinuous variations. Darwin's endless series of continuous variations is thus not substantiated by the

mathematical evidence which the author marshals.

The reviewer regrets that he is compelled to raise some more or less important criticisms of this long and exquisitely prepared volume. In his prefatory note Professor Thompson says that the book needs no preface since it is all preface. The reviewer would go further and point out that some of the chapters of which the volume is a preface have already been at least partially written but no reference to them is to be found. The vast evidence of the relation of hormones and similar agents to organic growth and form receives scant attention. Nor does the more recent study of the relation between molecular configuration and form and structure receive the hopeful treatment it merits. Moreover, the entire field of the genetics of form is passed by with little comment, and the work of the experimental embryologists and students of regeneration seems to have been overlooked entirely. These omissions in reality are faults of the subject rather than of the author; to expect even so learned a writer as Professor Thompson to cover this subject of growth and form in one volume would be unfair. For Life itself is Growth and Form.

The mathematics in the volume are almost exclusively the mathematics of statistics and geometry; the thermodynamic viewpoint is largely absent. While there are many references to comparatively recent papers, one is impressed by the fact that the greatest source of the author's factual materials and other references is in literature of an earlier date. No doubt American readers will be surprised at the omission of significant American literature and the absence of names familiar to every modern student of form determination. A less important criticism is that the Bibliography is presented in the form of footnotes; the usefulness of the book would have been greatly enhanced by the addition of a formal bibliography.

BIOLOGICAL SYMPOSIA, VOLUME VII: VISUAL MECHANISMS[1]
EDITED BY HEINRICH KLÜVER

Reviewed by SELIG HECHT
Columbia University

THIS is one of the fruits of the Fiftieth Anniversary Celebration of the University of Chicago. The Symposium on Visual Mechanisms held in September, 1941, has been expanded by the addition of four more contributions to make a volume of twelve papers, plus a foreword by Heinrich Klüver, the editor and organizer of the Symposium. The contents range over the problems of vision from the retina to the cerebral cortex: there are two papers on the structure of the retina, three on retinal physics and chemistry, and seven on the neurophysiology of vision.

One of the anatomical chapters is by Gordon L. Walls, who describes the rods and cones, their detailed makeup, their origins, their transformations in various vertebrates, their evolution, and their relation to vision in terms of the duplicity theory. The other chapter is by S. L. Polyak; it is concerned with the thirteen kinds of neurones in the retina,

[1] Lancaster, Pa.: Jacques Cattell Press, 1942. P. viii+322. $3.25.

their structure, and their complex but stable interrelations. These two papers are well illustrated and serve to put vision into its proper anatomical and zoölogical frame of reference.

The three chemical and physical papers are themselves quite varied. My own contribution deals with the energy required for us to see and with the absorption, distribution, and reception of these astonishingly small quantities of light. The paper by Arlington C. Krause is on the chemistry of visual purple, in which he gives the compositions and absorption spectra of a series of substances derived from visual purple. And George Wald has a chapter on the visual pigments of a variety of animals, showing their similarities and differences, especially in relation to their vitamins A and their retinenes and to their systematic positions in the animal kingdom.

Of the neurophysiological group of papers, four are descriptive and experimental, two are theoretical and speculative, while one is a bit of both. Of the factual papers, E. Gellhorn's on the influence of anoxia on vision describes the rapidly reversible changes in brightness discrimination, visual acuity, color perception, and afterimages produced by a decrease in the oxygen content of the respired air. The paper by T. J. Case examines the rhythmic brain potentials of over one hundred persons with supratentorial lesions and shows that a gross asymmetry of these alpha waves is a good diagnostic sign of occipital- or temporal-lobe lesions accompanied by loss of vision. In another paper, G. von Bonin, H. W. Garol, and W. S. McCulloch describe their work on mapping of the occipital lobe, which leads them to its division into a striate area, a parastriate area, and a preoccipital area, the first being concerned with vision directly and the other two with secondary influences. Heinrich Klüver's contribution summarizes the difficult and beautiful visual experiments with *Rhesus* monkeys before and after removal of the striate cortex. This work shows that, with the striate cortex intact, the animal is provided with a stable visual world, while without it the organism is a creature of the quantitative variation in light flux to which it is exposed.

The two theoretical papers are by K. S. Lashley and by W. H. Marshall and S. A. Talbot. Lashley's is an effort to provide a theory of cerebral organization essentially in terms of the changing pattern of stationary waves over the cortex produced by impulses originating at various parts of the cortex due to ocular stimulation. Marshall and Talbot are concerned with seeing how the various physiological mechanisms, from diffraction by the pupil to the properties of the cortex, work together to account for such complex visual functions as acuity perception and pattern illusions. A more restricted part of the same problem is considered by S. H. Bartley. He is interested in correlations between vision and neurophysiology, dealing not only with obvious parallelisms between sensation and neural activity but with more subtle problems involving spatial and temporal relations of phenomena like brightness perception and flicker fusion.

It is apparent that vast information and much thought have gone into the preparation of this book. In a composite book there is a tendency for each author to pack his contribution so that the density of thought per unit space is high —so high that occasionally it obstructs the free flow of ideas. This is occasionally true here, and it shows itself when an author assumes too much preparation

and knowledge on the part of the reader; the result is that a few of the papers are hard to understand without extra study. In the main, however, the contributions are well written and easy to follow.

The physiology of vision has not had the general interest of biologists, because it is considered a narrow field, of importance only to those working in it. That this is erroneous will be apparent to anyone who reads even a few of the papers. Here he will find many general problems of biology, not only those concerned with the relation between sense organ and brain organization, but those dealing with the interactions of organisms and their environment and extending into systematics and evolution.